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Steroid-C^{14} Metabolism in Bile Acid and Cholesterol-Treated Mice after Injection of Mevalonic Acid-2-C^{14}.* (26994)

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In the study of cholesterol and bile acid metabolism, use of single injections of isotopically labeled cholesterol or cholesterol precursors coupled with fecal steroid analysis has been incompletely investigated. Siperstein and coworkers(1,2) gave single intravenous and oral doses of cholesterol-4-C^{14} to rats and found that in a 2-week interval from 50 to 90% of metabolized cholesterol was excreted in the fecal bile acid fraction. Similar results were obtained by Danielsson(3), using intraperitoneal injections of cholesterol-4-C^{14} emulsions. Kummerow *et al*(4) demonstrated that the pattern of steroid metabolism in the chicken differs from that in the rat only in respect to rates of synthesis and excretion. On the other hand, Frantz *et al*(5) could not confirm these findings in human studies in which, following a single injection of cholesterol-C^{14}, 80% of the fecal C^{14} steroids were excreted in the non-saponifiable fraction.

In the present experiment, we have determined the pattern of fecal steroid-C^{14} excretion as a function of time after single intraperitoneal injections of mevalonic acid-2-C^{14} in normal, bile acid and cholesterol treated mice. The results demonstrate some of the advantages and limitations of such an approach in the study of cholesterol metabolism under various conditions.

Methods. Forty Webster strain female albino mice were placed on a cholesterol free diet(6) for 1 week, then divided into 5 groups and supplemented as shown in Table I. After 3 weeks, each animal received intraperitoneally 1 μc of mevalonic acid-2-C^{14} in saline (1.31×10^6 counts/min.). Each mouse was housed in a metabolism cage and feces collected at 24 hour intervals for the 7-day ex-

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TABLE I. Fecal Steroid-C^{14} Excretion as a Time Function, Following Injection of Mevalonic Acid-2-C^{14}

	Control		Cholic Acid*		Cholesterol + Cholic†		Litho-cholic acid*		Hyodeoxy-cholic acid*	
Days	$\alpha + \beta$ Sterols	Bile acid	$\alpha + \beta$ Sterols	Bile acid	$\alpha + \beta$ Sterols	Bile acid	$\alpha + \beta$ Sterols	Bile acid	$\alpha + \beta$ Sterols	Bile acid
	Counts/min./mouse/24 hr									
1	7264	19810	10643	10705	14920	7522	9295	7025	20665	11381
2	5755	2187	5600	3248	6134	1278	7403	3257	15211	2055
3	3326	1683	5004	1492	4034	843	5980	2236	10698	1494
4	3061	1818	3755	1044	3732	158	5584	1670	9171	1340
5-7	2572	2459	1846	2030	3127	261	4993	1877	7621	1148
Total Excretion	21978	27957	26848	18519	31947	10062	33255	16065	63366	17418

* Diet supplemented with .5% of bile acid.
† " " " .5% cholic acid + 1% cholesterol.

perimental period, after which the animals were sacrificed, livers removed, and both livers and carcasses quick frozen.

The feces samples were continuously extracted with hot ethanol for 48 hours. Aliquots of the ethanol extract containing the total steroids, were made strongly basic by addition of 7 N NaOH and heated in an autoclave at 15 lb pressure for 3 hours. The nonsaponifiable fraction was extracted with petroleum ether, and aliquots removed for determination of total β sterols and C^{14} activity of the $\alpha + \beta$ sterols. The aqueous residue was acidified and fatty acids extracted with petroleum ether and discarded. The remaining acidified aqueous fraction was extracted with ethyl ether. Aliquots of this bile acid containing extract, were plated and counted.

Liver and carcass cholesterol and β-sterol-C^{14} were determined by saponification of the respective tissues in alcoholic KOH, followed by petroleum ether extraction. Aliquots of the nonsaponifiable fraction were evaporated to dryness, taken up in alcohol-acetone (1:1) and precipitated with digitonin. β-Sterol-C^{14} was determined by filtration and washing of the digitonide precipitate on a filtration tower. All C^{14} determinations were made in an automatic windowless flow counter. All cholesterol assays were done according to the method of Sperry and Webb(7).

Results and discussion. Control. In normal mice the distribution of excreted fecal-C^{14} activity in the first 24 hours was 73% bile acids, 27% $\alpha + \beta$ sterols (Table I). This distribution was reversed in the 2nd 24-hour period (28% bile acids, 72% neutral sterols), and remained more or less fixed during the following collection periods. Thus, the overall pattern of excreted steroid-C^{14} activity in mice during the 7-day experimental period was 56% bile acids, 44% neutral sterols. Since there was a definite reversal in the excretory pattern after the first 24-hour collection, it is possible that shortly after injection most of the activity appears in the bile acid fraction. Although no experimental data are available, it seems likely that this phenomenon may be explained by the rapid turnover rate in liver cholesterol coupled with the relatively slow rate in extrahepatic tissues, since in normal animals the neutral fecal sterols are mainly extrahepatic in origin(3,8). Of the injected mevalonic acid-2-C^{14} activity, some 3.8% was incorporated in fecal C^{14}-steroids excreted during the 7-day period (Table II).

Cholic acid. A strong deterrent effect of cholic acid and its conjugates on the conversion of cholesterol to bile acids, in mice with accumulated liver cholesterol, has been demonstrated previously(8,9). Experiments of others have shown a reduction in production of bile acids in bile fistula rats receiving taurochenodeoxycholic acid(10). Both of these approaches utilized an abnormal situation, while the present experiment was designed to test the effect of cholic acid on bile acid formation in the intact mouse. The time-pattern of steroid-C^{14} excretion in cholic acid-treated mice was similar to that in untreated animals, but total bile acid synthesis

TABLE II. Effect of Bile Acids and Cholesterol on Fecal and Tissue Steroid and Steroid-C^{14} Levels.

	Control	Cholic acid	Cholesterol + cholic acid	Lithocholic acid	Hyodeoxycholic acid
			Counts/min./mouse/24 hr		
Total steroid-C^{14} excreted in feces	49935	45367	42009	49320	80784
β-Sterol-C^{14} remaining in liver	9399	21090	38954	8747	5989
β-Sterol-C^{14} remaining in carcass*	45539	44864	32621	44260	46978
Total C^{14}-activity accounted for	104873	111321	113584	102327	133751
			mg/24 hours		
Fecal β-sterol	1.93 ± .50	2.79 ± .54	—	4.34 ± .70	10.3 ± 1.90
			mg/g		
Liver cholesterol	4.52 ± 1.56	7.53 ± 2.54	48.0 ± 11.4	4.35 ± 1.44	2.64 ± .44
Carcass* "	2.16 ± .50	2.30 ± .39	2.93 ± .25	1.80 ± .43	2.00 ± .28

* Carcass weight = Body weight minus liver weight.
± numbers are standard deviations.

during the 7-day experimental period was less (Table I). There was a significant inhibition (50%) of bile acid excretion in the first 24 hours, but in subsequent periods there was no inhibition. Analysis of liver and carcass β-sterol-C^{14} activity at the end of 7 days revealed a significant retention of β-sterol-C^{14} activity in livers of cholic acid-treated animals (Table II). No significant difference in carcass activity was observed, therefore the deterrent effect of cholic acid must be confined to the liver. Total steroid-C^{14} synthesized was similar in control and cholic acid-treated mice. Since cholic acid retards hepatic cholesterol synthesis 75% in mice(9), it is impossible to assess the effect of cholic acid on cholesterol synthesis rates from an experiment of this type.

Cholesterol plus cholic acid. Cholesterol plus cholic acid increased the output of C^{14}-labeled $\alpha + \beta$ sterols during the 7-day collection period, and especially enhanced the output during the initial 24 hours. The rate of appearance of C^{14}-activity in the fecal bile acid fraction was greatly retarded throughout the collection periods (Table I). Because of this retarded bile acid-C^{14} synthesis, mice in this group retained much more of the liver β-sterol-C^{14} activity at the end of 7 days (Table II) than the controls, yet their total fecal steroid-C^{14} excretion was *not* significantly lower. This is explained by the fact that in this group a greater proportion of the fecal C^{14}-activity appeared in the $\alpha + \beta$ sterol fraction, which compensated for the loss in excreted bile acid-C^{14} (Table I).

The enhanced loss of carcass β-sterol activity, which appeared in the fecal $\alpha + \beta$ sterol fraction, can not be explained by the data of the present experiment. However, it seems probable that this loss was due to an increased rate of carcass endogenous-exogenous cholesterol turnover caused by dietary cholesterol.

Hyodeoxycholic acid. Hyodeoxycholic acid altered the time-pattern of fecal C^{14} activity (Table I). There was a large absolute increase in $\alpha + \beta$ sterol-C^{14} excretion at all time intervals, reflecting the increased fecal β-sterol excretion (Table II). A similar increase has been shown to occur in the golden hamster on the first day of hyodeoxycholic acid feeding(11). The present data (Table II) show that at termination the livers of hyodeoxycholic acid-treated mice contained much less β-sterol-C^{14} than livers of controls, while carcass β-sterol-C^{14} was about the same in these 2 groups; therefore, it may be concluded that fecal sterols in hyodeoxycholic acid-treated mice originate in the liver and are excreted via the bile duct. It is conceivable that liver cholesterol could first be transferred to extrahepatic tissues and then be excreted; however, one would expect a definite change in carcass cholesterol-C^{14} if this were true. Direct proof awaits bile duct cannulation experiments in a larger species. If one considers that hepatic cholesterol synthesis is increased some 7-fold in hyodeoxycholic acid-treated mice(12) and that there is a 5-fold increase in β-sterol excretion (Table II), the inadequacy of using single injections of mevalonic acid for detection of changes in cholesterol synthesis rates is apparent (hyodeoxycholic acid-treated: 133,751 counts/min.; controls: 104,873 counts/min.). Some investigations may have failed to reveal changes in synthesis rates from mevalonic acid, while demonstrating large increases or decreases in cholesterol synthesis from acetate-1-C^{14}, because of the use of limiting concentrations of mevalonic acid.

Lithocholic acid. The effects of lithocholic acid on mouse liver cholesterol levels, synthesis rates(12), and blood cholesterol levels(13) were similar to, but less extensive than, the effects of hyodeoxycholic acid. The data in Tables I and II indicate that the mechanism of action of these bile acids is similar, although the detailed and overall effects of lithocholic acid are smaller.

Considering the results of the foregoing experiments *in toto,* it is evident that under normal circumstances mobilization of mouse liver cholesterol depends upon the ability of this organ to convert cholesterol to bile acids. However, hyodeoxycholic and lithocholic acids in some unknown way enable the mouse liver to bypass this requirement and excrete cholesterol directly. Direct excretion was shown to apply not only to accumulated liver cholesterol(8) but also to normal hepatic cholesterol (Table II).

This study makes it clear that the time-pattern of fecal steroid-C^{14} excretion is greatly influenced by the experimental conditions. In almost all cases observed differences were due to alterations in hepatic cholesterol metabolism.

Summary. The effects of bile acid and cholesterol on the time-pattern of fecal steroid-C^{14} elimination was investigated in mice following a single injection of mevalonic acid-2-C^{14}. In controls, excreted bile acid-C^{14} assayed 3 times sterol-C^{14} during the first 24 hours. This ratio reversed during the 2nd 24-hour period, and remained constant for the remainder of the 7-day experiment. Cholic acid depressed bile acid-C^{14} excretion, but failed to alter the time pattern of fecal sterol-C^{14} elimination. Cholesterol plus cholic acid depressed bile acid-C^{14} and increased $\alpha + \beta$ sterol-C^{14} excretion throughout the experiment. Hyodeoxycholic acid increased sterol-C^{14} excretion (shown to be of hepatic origin), had little effect on bile acid-C^{14} excretion, and increased total steroid-C^{14} eliminated. Lithocholic acid had a similar though smaller effect. After sacrifice, the livers of cholic acid-treated mice had twice the β-sterol-C^{14} concentration found in livers of controls, while those of mice treated with cholesterol plus cholic acid had β-sterol-C^{14} levels 4 times as high. Livers of lithocholic and hyodeoxycholic acid-treated animals showed reduced β-sterol-C^{14} levels. Carcass β-sterol-C^{14} levels were unaffected by any of the bile acids, but were lowered by the combination of cholesterol and cholic acid. The total amount of sterol-C^{14} synthesized from injected mevalonic acid was similar in all cases.

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Decreased or Absent ACTH-Like Activity of Blocked Synthetic Tricosapeptide.* (26995)

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A synthetic tricosapeptide synthesized by Hofmann *et al.*(1) which duplicates the first 23 amino acids, beginning with the N terminus, of naturally occurring ACTH(2-5) has been shown to increase urinary excretion of 11-oxysteroids and of 17-ketosteroids in adult male prisoners and in a patient with scleroderma(6). Trials of a variant of this molecule with certain groups blocked by a variety of means have been undertaken in a search for steroidogenic, electrolyte, carbohydrate or other effects. In contrast to the ACTH-like activities of the unblocked parent tricosapeptide this molecule appears to be less active or inert.

Materials and Methods. Fig. 1 depicts the molecular structure of the active tricosapeptide and its blocked congener. In the deblocked molecule R, R′, and R″ refer to H^+, OH^-, and H^-, respectively; in the blocked molecule R, R′, and R″ represent acetyl, amino, and formyl groups, respectively(7,8).

The blocked tricosapeptide amide, dissolved in the gelatin-saline base employed by Armour and Co. in preparation of commercial Acthar gel, was administered in aliquots which contained 6.7 mg of material. This dosage was therefore comparable to that of the unblocked tricosapeptide (6.4 mg of deblocked peptide with ACTH-like activity) per injection. Four healthy male adult prisoners served as subjects. Blood and sera obtained in the fasting state prior to and during the series of injections were analyzed for levels of sugar, NPN, CO_2, chloride, sodium, potassium, total protein, albumin, globulin, calcium, inorganic phosphorus, protein-bound iodine, total and alpha- and beta lipoprotein, cholesterol, phospholipids, triglycerides and non-esterified fatty acids (NEFA) by methods in regular use in these laboratories(6). Urinary excretion in refrigerated 24-hour specimens of 11-oxysteroids (Porter-Silber chromogens), 17-ketosteroids, and pressor amines was normal before and during therapy. Possible effects upon carbohydrate metabolism were sought by means of oral glucose tolerance tests (1.75 g of glucose per kilo of body weight) and intravenous

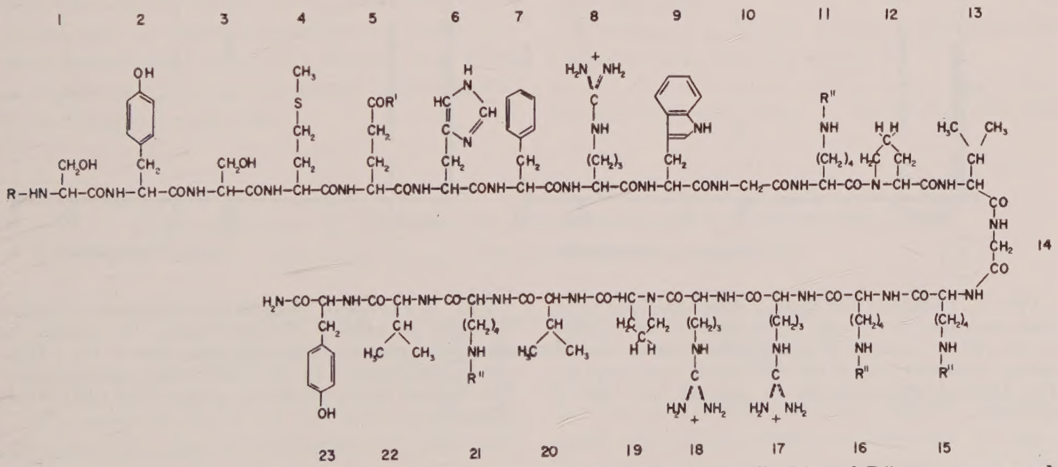

FIG. 1. Structure of Deblocked and Blocked Synthetic Tricosapeptide. In the deblocked molecule R, R′, and R″ refer to H^+, OH^-, and H^-, respectively; in the blocked molecule R, R′, and R″ represent acetyl, amino, and formyl groups, respectively.

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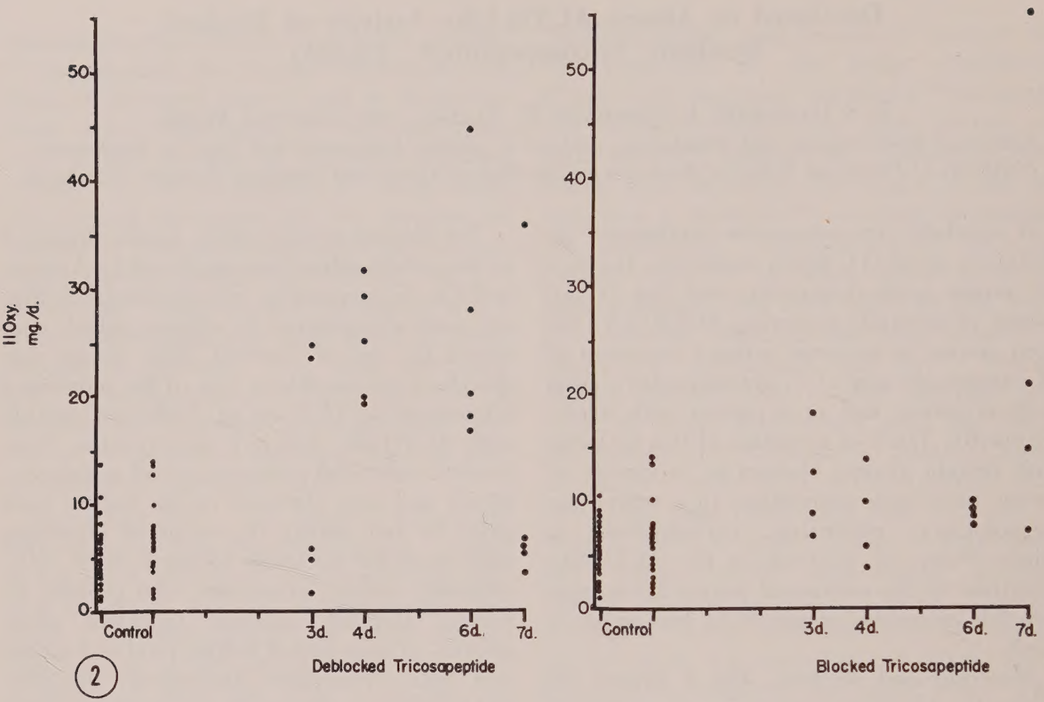

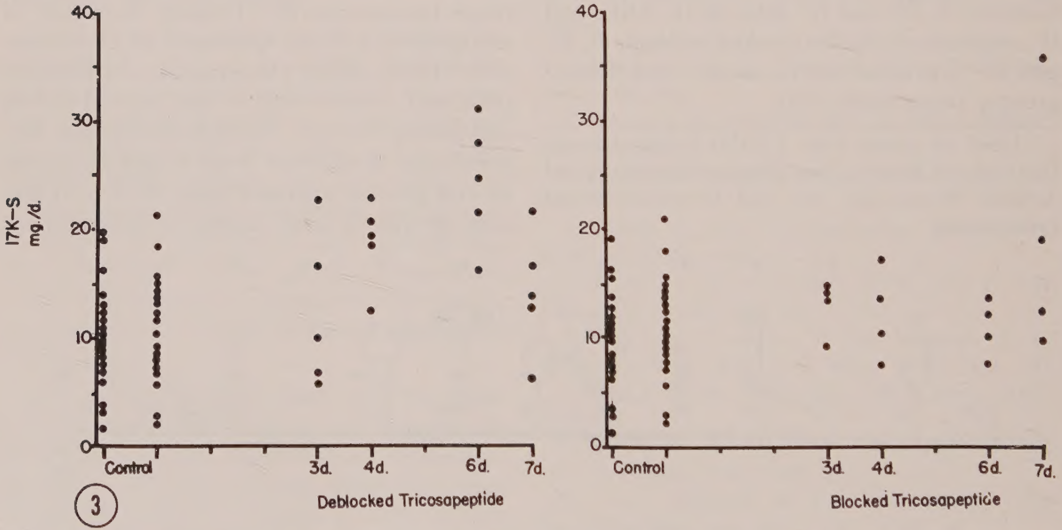

FIG. 2. Failure of the blocked tricosapeptide to induce increases in urinary 11-oxysteroids. In contrast to the rise in urinary 11-oxysteroids observe the following administration of the deblocked tricosapeptide. The blocked tricosapeptide in general had little if any effect.

FIG. 3. Failure of blocked tricosapeptide to induce increases in urinary 17-ketosteroids. In contrast to the rise in urinary 17-ketosteroids observe the following administration of the deblocked tricosapeptide. The blocked tricosapeptide in general had little if any effect.

TABLE I. Mean Change in Serum Electrolytes

		CO_2 (meq/L)	Cl (meq/L)	K (meq/L)
Tricosapeptide (deblocked) 5 subjects	6.7 mg* peptide/d/6d	+1.1	+1.10	−.44
	6.7 mg peptide/d/7d	+ .9	−1.54	−.64
Tricosapeptide (blocked) 4 subjects	6.4 mg peptide amide/d/6d	−1.2	+2.82	−.60
	6.4 mg peptide amide/d/7d	−1.3	+1.88	−.28

*equals 160 subcutaneous adrenal ascorbic acid depleting units.

insulin tolerance tests (0.1 unit of commercial regular insulin per kilo of body weight).

Results. There were no increases in blood pressure or body weight nor did any of the subjects develop Cushingoid manifestations. The deblocked tricosapeptide increased the urinary 11-oxysteroids and to a lesser extent the 17-ketosteroids while administration of the blocked congener, with the exception of one specimen in one patient, did not produce these changes. The sole exception occurred after the seventh injection of the blocked tricosapeptide (Figs. 2, 3).

The serum electrolytes showed a slight rise in CO_2 and a decrease in chloride and potassium when the deblocked tricosapeptide was administered, in keeping with changes induced by commercial ACTH. With the blocked tricosapeptide only the serum potassium values decreased (Table I).

The glucose and insulin tolerance tests were not altered by the blocked tricosapeptide whereas the deblocked polypeptide may have manifested a slight hyperglycemic and perhaps anti-insulin effect(6). Neither was there any pattern of change in serum lipids elicited by the blocked molecule.

Summary. Substitution of acetyl for hydrogen in the NH_2 group of the N-terminal serine, NH_2 for OH^- in the carboxyl of glutamine in position 5, and formyl for an amino hydrogen of lysine in positions 11, 15, 16, and 21 decreases or eliminates the ACTH-like activity of a synthetic tricosapeptide which duplicates the first 23 amino acids of naturally occurring ACTH.

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Repopulation of Thymus by Immunologically Competent Cells Derived from Donor Marrow. (26996)

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The cortical region of the thymus becomes atrophic following X-irradiation(1); however, the thymus recovers weight rapidly in irradiated mice that receive an injection of isologous(2,3) or parental bone marrow(2). This recovery may result from repopulation of the thymus by implanted cells, although Kaplan

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* Operated by Union Carbide Corp. for U. S. Atomic Energy Commission.

et al.(4) reported that 6 of 9 lymphomas developing in the thymuses of irradiated (C57BL × BALB/c) F_1 mice that had received parental bone marrow did not contain cells derived from the injected marrow. Moreover, the thymus did not return to normal weight in irradiated mice injected with homologous or heterologous bone marrow(2). Such observations suggest that the thymus may not be repopulated by parental, homologous, or heterologous marrow cells, although it has been shown that such marrow will repopulate other hematopoietic tissues of irradiated mice(5). On the other hand, Gengozian *et al.*(6) found that thymuses of lethally irradiated mice that received an injection of rat (heterologous) bone marrow did contain cells identified cytologically and serologically as rat cells.

Additional information as to the type of cells in the thymus of radiation chimeras seemed desirable in view of these apparent differences. Hence, an experiment was designed to determine the preponderant type of lymphopoietic cell (donor or recipient) in regenerated thymuses of irradiated F_1 hybrid mice given bone marrow cells of parental origin. The results of this study are reported herein.

Materials and methods. Four stocks of mice were used in this investigation: 1) C57BL/Cum, 2) 101/Cum, 3) (C57BL/Cum × 101/Cum)F_1, and 4) (C57BL/6 Cum × DBA/2 Cum)F_1. Stocks will be referred to as C57BL, 101, B1F_1, and B6D2F_1 mice, respectively. Age of the mice varied from 4 to 6 months. Approximately equal numbers of each sex were used, except that all of the B6D2F_1 mice were males.

The scheme of the experiment was as follows:

X-irradiated controls. B1F_1 and B6D2F_1 mice were given 650 r to determine the effect of X-rays† alone on their survival.

X-irradiated, thymus-injected mice. A single dose of 650 r, which was sublethal for B1F_1 mice and a LD 13/30 days dose for B6D2F_1 mice, was given 1 to 4 hours before such mice received an intraperitoneal injection of a thymus cell suspension. Thymus glands were excised from C57BL, 101, and B1F_1 donor mice. A cell suspension was made by cutting thymus glands of 5 donors into small pieces with scissors and by flushing the fragments several times through an 18- or 20-gauge hypodermic needle. The equivalent of one donor thymus was injected intraperitoneally into each of the 650 r X-irradiated recipients.

X-irradiated, regenerated thymus-injected mice. Thirty million bone marrow cells of strain C57BL or 101 mice were injected intravenously by tail vein into irradiated C57BL, 101, or B1F_1 mice within 4 hours after receiving a lethal dose of X-rays; a dose of 950 r was given to B1F_1 and 101 mice, and 850 r to C57BL mice. Thymus glands of such bone marrow recipients were excised 21 days later; thymus cell suspensions were prepared by the above method, and the cells were injected intraperitoneally into 650 r X-irradiated F_1 hybrid mice.

Thymuses of a few lethally irradiated, bone marrow-treated mice were fixed in Zenker-formol solution. Lymph nodes, spleen, and bone marrow of a few "X-irradiated, thymus-injected mice" and "X-irradiated, regenerated thymus-injected mice" were also fixed in Zenker-formol. Hematoxylin-eosin-stained sections of these tissues were prepared for histological examination.

Results. An X-ray dose of 650 r was sublethal for B1F_1 mice, and the survival of such mice was not altered by an injection of isologous thymus cells (Table I, lines 1 and 2). In contrast, 83% of 650 r-irradiated B1F_1 mice died within 90 days after receiving C57BL thymus cells and 50% died within 90 days after receiving 101 thymus cells (Table I, lines 3 and 4). Regenerated thymus tissue of lethally irradiated, isologous bone marrow-treated C57BL or 101 donors killed sublethally irradiated B1F_1 mice as, or more, effectively (Table I, lines 5 and 6) as the thymus cells of non-treated parental strain mice.

A second series of experiments was done to assay whether thymus cells in lethally irradiated B1F_1 mice that had received an injection of parental marrow cells were of recipients or donor origin.

For this series, a B6D2F_1 hybrid having only the parental donor strain in common with the B1F_1 bone marrow recipients was used. As

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† Physical factors were: 300 kvP; 20 ma; 4.75 mm Be inherent filtration, 3.0 mm Al added filtration; 93.7 cm target-mouse distance; output ~90 r/min; HVL, 0.558 mm Cu.

TABLE I. Survival of Sublethally Irradiated F_1 Hybrid Mice Injected with Thymus Cells.

Donor Strain	Recipient Strain	Recipient No.	Survival (%) Days after treatment 10	30	60	90
1. —	B1F_1	48	100	100	100	100
2. B1F_1	idem	48	98	98	98	98
3. C57BL	"	48	85	48	25	17
4. 101	"	48	100	69	58	50
5. C57BL/C57BL*	"	30	93	37	23	17
6. 101/101*	"	64	94	36	23	9
7. —	B6D2F_1	52	100	87	85	83
8. B1F_1	idem	24	96	92	92	92
9. C57BL	"	24	96	0	0	0
10. B1F_1/C57BL†	"	56	100	5‡	0	0

*Isologous bone marrow chimeras (see text). †Parent-F_1 bone marrow chimeras (see text).
‡All 30-day survivors were dead 40 days after treatment.

indicated in Table I (lines 7 and 8), 650 r of X-rays was lethal for 17% of the B6D2F_1 hybrids, and an intraperitoneal injection of B1F_1 thymus cells did not further affect survival. However, B6D2F_1 mice given 650 r of X-rays and injected with C57BL thymus cells died within 30 days after treatment (Table I, line 9). Similarly, irradiated B6D2F_1 hybrids that received regenerated thymus cells from lethally irradiated C57BL marrow-treated B1F_1 donors died within 40 days after treatment (Table I, line 10). Although C57BL and C57BL/6 mice are of different strains, their major histocompatibility factors are similar, and C57BL thymus cells did induce a wasting disease in irradiated B6D2F_1 mice.

Pathological findings. Histological examination showed that much of the thymus cortex had regenerated within 21 days in B1F_1 mice given parental bone marrow. B6D2F_1 mice given regenerated thymus tissue from C57BL bone marrow-treated B1F_1 donors showed granulomatous reticular cell hyperplasia and hypertrophy in the lymph nodes, splenic white pulp, and Peyer's patches of the intestine. These active granulomatous foci were similar to those observed in sublethally irradiated B1F_1 mice injected with thymus cells from normal C57BL or 101 donors and in the irradiated B6D2F_1 mice injected with C57BL thymus cells. The histological findings are also similar to those occurring in sublethally irradiated F_1 mice injected with parental spleen cells(7)‡.

Discussion. The conclusion drawn from this study is that the thymus in a lethally irradiated F_1 hybrid given parental marrow is repopulated 21 days after treatment by donor marrow cells. A more rapid recovery of thymus weight in irradiated F_1 hybrids receiving parental bone marrow than in those not receiving marrow(2) suggested that the thymus was actually repopulated, even though some of the thymic lymphomas that developed later in such mice were of host type(4). Our study was made using the newly regenerated thymus, whereas the thymic lymphomas tested by Kaplan *et al.*(4) appeared after a latent period of more than 80 days. The type of thymus cells in such late radiation chimeras may not be the same as 21 days after bone marrow transplantation since grafts of parental marrow may regress within 90 days or longer after implantation(8). The mortality in 650 r-irradiated F_1 mice injected with parental lymphoid cells is attributed to genetically determined histocompatibility differences between tissues of the recipient and donor; *i.e.*, immunologically competent cells of parental strain mice produce antibodies against tissue antigens of the F_1 hybrid recipient(9,10,11). Pathological findings support the view that the sublethally irradiated B1F_1 recipients of parental strain thymus cells died as a result of an immunological reaction, a conclusion reported previously by Kaplan and Rosston(12). Studies of Congdon and Duda(13) also suggest that thymus cells injected into irradiated mice may give rise to antibody and plasma cells.

Summary. Many of the sublethally irradiated B1F_1 and B6D2F_1 mice injected intraperitoneally with thymus cells from parental strain donors died within 90 days, owing presumably to an immunological reaction by the

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‡ The author is grateful to Dr. G. E. Cosgrove for his interpretation of the histological preparations.

implanted cells against the recipient. Likewise, regenerated thymus tissue of lethally irradiated B1F$_1$ mice injected with C57BL bone marrow cells induced a fatal wasting disease on implantation in sublethally irradiated B6D2F$_1$ mice. It was concluded, therefore, that regenerated thymus tissue in lethally irradiated B1F$_1$ mice injected with C57BL bone marrow was repopulated by immunologically competent cells derived from the donor marrow.

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Effect of Ribonuclease Upon Immunologically Active Cells.* (26997)

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Brent *et al*(1) have demonstrated that lymphoid cells of homograft sensitized animals provoke a cutaneous reaction of the delayed type (the "transfer reaction") when injected intradermally in the antigen donor. The specificity of this reaction is consistent with 2 possibilities: that the transfer reaction is dependent on "cell-bound antibodies" or "endoantibodies" or that humoral antibodies are produced in such small quantities that their effect can be detected only in the immediate vicinity of the cells which produce them(2). The character of the reaction itself, which is an indurated inflammatory lesion, implies either that host cells infiltrate the injection site or that the transferred cells increase in mass by multiplication and/or synthesis of new cytoplasmic material. All these features require an intact protein synthesis.

In the present experiments we have applied a biochemical technique to this immunological model to evaluate the role of protein synthesis by immunologically active cells in producing the reaction. Ribonuclease provides a useful tool for investigating this problem. Allfrey *et al*(3) demonstrated in experiments with microsomes that the integrity of ribonucleic acid may be deleteriously affected by ribonuclease with a resulting inhibition of protein synthesis, and Brachet(4), and Ledoux and Baltus (5) have shown that ribonuclease penetrates many living cells and is able to slow protein anabolism without killing the target cells.

Materials and Methods. Dutch rabbits were immunized with slices of New Zealand rabbit ear skin implanted subcutaneously. Ten days later draining lymph nodes were recovered, minced in cold Hanks' solution and a cell suspension prepared. Active ribonuclease (5 × crystallized, Nutritional Biochemicals Corp.) or ribonuclease inactivated by heat (30 min at 100°C) were added to aliquots containing equal numbers of cells to give a final concen-

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† Post-Sophomore Fellow, Nat. Inst. of Health.

TABLE I. Transfer Reaction with Lymphoid Cells Exposed to Ribonuclease.

Concentration of ribonuclease	Time of incubation at 37°C	No. of living cells per 0.1 ml (million)		Skin reaction		
(mg/ml)	(min.)	before incubation	after incubation	24 hr	48 hr	72 hr
		Rabbit 1				
4	0	2.5		sl.ery.	+	+
"	60	"	2.1	" "	0	0
in.RNAse*	0	"		++	++++	+++ N
"	60	"	1.9	++	+++	+++ N
—	—	2.5 million cells only (control)		++	+++	++++ N
		Rabbit 2				
4	0	2.5		sl.ery.	+	+
"	60	"	2.2	" "	+	0
in.RNAse	0	"		+	+++	+++ N
"	60	"	1.7	+	+++	+++ N
—	—	2.5 million cells only (control)		++	++++	+++ N
		Rabbit 3				
4	0	2.5		+	+	±
"	60	"	2.4	0	0	0
in.RNAse	0	"		+	+++	+++
"	60	"	2.1	+	++	+++
—	—	2.5 million cells only (control)		++	+++	++++

*in.RNAse = ribonuclease inactivated by heat; sl.ery. = slight erythema; ±, +, ++, +++ and ++++ represent different degrees of induration; N = necrosis.

tration of 4 mg/ml. The cells were injected intradermally in the original New Zealand antigen donor immediately after addition of active or inactivated ribonuclease, and again after 60 min of incubation at 37°C. Approximately 2.5 million viable cells were injected in 0.1 ml. Cells were counted at zero time and again after 60 min of incubation using the trypan blue method as a measure of cell viability. The sites of cell injection were examined daily for erythema, induration and necrosis, and the skin reactions graded on an arbitrary scale ranging from ± to ++++.

Results. The results (Table I) suggest that ribonuclease directly affects the immunological activity of lymphoid cells from a sensitized rabbit, depriving them of their ability to produce the transfer reaction when injected into the skin of the antigen donor. The fact that cells injected immediately after addition of ribonuclease still give positive reactions indicates that the action of this enzyme is directed against injected cells, not against host cells migrating to the site of reaction. This inference is further supported by the fact that cells incubated with ribonuclease and then washed to remove the enzyme before injection into the donor lose the ability to produce a transfer reaction. Cells treated with biologically inactive enzyme retain their immunological competence and give a transfer reaction of the same intensity as nontreated (control) cells. The exposure of cells to 4 mg/ml of ribonuclease for one hour at 37°C does not kill the cells, as judged by the trypan blue method or subsequent survival of treated cells in Millipore filter chambers. Smears of treated cells stained with methyl-green-pyronin showed a recognizable decrease in cytoplasmic basophilia of certain cell types as compared with smears of control cells.

Discussion. Although the exact mechanism of ribonuclease activity upon living cells is not known, it has been postulated that this enzyme acts by breaking down soluble ribonucleic acid or by forming a complex with this acid and thus inactivating it(6). A variety of cells treated with ribonuclease show abnormal incorporation of amino acids into protein molecules, impairment of protein synthesis, and depression of cell growth and mitotic activity (cf. 7). It is not unlikely that ribonuclease exerts a similar effect on the lymphoid cells used in our experimental model. Since our data show that the specific activity of immune lymph node cells is inhibited, it is probable that the transfer reaction is ultimately bound to the protein anabolism of immunologically active cells, *i.e.* intact protein synthesis is essential for expression of the reaction. However, it remains to be determined whether this is an increased synthesis of specific protein or antibody in situ ("local secondary specific protein or antibody response") on second contact of immunologically active cells with specific antigen, or the increased synthesis of protein accompanying increased cell size and/or proliferation.

Summary. Incubation of homograft sensitized lymphoid cells with ribonuclease inhibits their ability to produce the "transfer reaction" on intradermal injection into the antigen donor. Ribonuclease in the concentration used does not kill the cells as determined by trypan blue staining and culture in Millipore filter chambers. The data indicate that intact protein synthesis of transferred immunologically active cells is essential for the expression of the "transfer reaction."

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Effect of Solvents on Eosinophile-Stimulating Substance of Erythrocyte Stroma.* (26998)

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It has been shown that blood is capable of exciting in animals a peritoneal reaction which in its later stages is characterized by the appearance of eosinophiles in definitely elevated proportions. It was then established that the component of blood responsible for this reaction was neither plasma nor hemoglobin but the stroma and membranes of erythrocytes (1,2).

The present studies were undertaken as the first steps toward isolation and identification of the eosinophile-stimulating substance (E.S.S.), if such exists.

Materials and Methods. Erythrocyte stroma was prepared as previously described.

Procedure 1. 2.5 ml of stroma was shaken repeatedly in 7.5 ml ether at room temperature, allowed to stand over night, reshaken and allowed to sediment. After filtration the soluble fraction was evaporated in partial vacuum to dryness. The residue was suspended with repeated shaking in sterile 0.7% saline to make 10 ml, and 1 ml of the suspension was injected into the peritoneal cavity of each of 10 male albino mice. The insoluble portion was likewise suspended in 0.7% sterile saline to make 10 ml, and 1 ml was injected intraperitoneally into each of 10 male albino mice.

Procedure 2. 2.5 ml of erythrocyte stroma was suspended in 7.5 ml petroleum ether and was treated in the same manner as described in procedure 1. One ml of the hexane soluble fraction suspended in 0.7% sterile saline was injected intraperitoneally into each of 10 male albino mice, and the insoluble portion was similarly injected.

Procedure 3. This experiment was carried out in the same way as procedure 1, except that chloroform was used as the initial extraction agent. Both soluble and insoluble fractions were resuspended and injected as before.

Procedure 4. Except for the use of acetone as the initial solvent this procedure was iden-

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tical with procedure 1. Resuspended fractions were treated and injected into mice in the identical way.

Procedure 5. Alcohol 95% was employed as the initial extraction agent. All other steps were carried out as in procedure 1.

Procedure 6. Equal parts of 95% alcohol and ether were used as the initial extraction agents. All other steps were the same as in procedure 1.

Procedure 7. 2.5 ml of erythrocyte stroma was shaken repeatedly with N/10 HCl. Complete solution occurred. When N/10 NaOH was added a precipitate occurred at pH below 7, presumably the iso-electric point of certain proteins. The pH was brought to 7.0, the material centrifuged and filtered. Soluble and insoluble fractions were reconstituted to 10 ml, and mice were then injected intraperitoneally, 10 with 1 ml of the soluble and 10 with 1 ml of the insoluble portions.

Procedure 8. 2.5 ml erythrocyte stroma was treated with 7.5 ml N/10 NaOH. Complete solution occurred, and adjustment with N/10 HCl to pH 7 produced no precipitation. One ml of the neutralized solution was injected intraperitoneally into each of 10 mice.

Procedure 9. 2.5 ml of stroma was shaken with 7.5 ml 10% urea in distilled water. Complete solution took place. One ml of the urea solution was then injected intraperitoneally into each of 10 male albino mice.

Procedure 10. 2.5 ml of stroma was suspended in 7.5 ml, 0.7% NaCl solution. The mixture was placed for one hour in a water bath held at 50°C. The material was then filtered and soluble and insoluble fractions brought up to 10 ml with 0.7% NaCl solution. Ten mice each received intraperitoneal injections of 1 ml of the insoluble suspension and 10 received each 1 ml of the solution.

Procedure 11. 2.5 ml stroma was boiled for one hour in 7.5 ml of 0.7% NaCl solution. The material was filtered, and the soluble portion brought up to 10 ml with 0.7% NaCl solution. Each of 10 male albino mice received 1 ml of the solution intraperitoneally.

Procedure 12. 2.5 ml stroma was digested at 37° with 7.5 cc 1% trypsin solution. Almost all material was dissolved. The solution was filtered, and the volume of filtrate brought to 10 ml. The undissolved residue was suspended in 10 ml 0.7% NaCl. One ml of filtrate was injected into each of 10 albino mice. One ml of suspension was injected into each of 10 animals.

Procedure 13. 2.5 ml stroma was digested with 7.5 ml 5% pepsin for 18 hours at 37°. The filtrate and residue were treated as in procedure 11.

Procedure 14. This procedure was identical with procedures 12 and 13, except that 1% caroid was employed as digestant. Mice were injected as before.

Procedure 15. Except that papain 0.1% was employed as the enzyme, this procedure was identical with the previous ones.

Procedure 16. Stroma was digested with 1% ptyalin.

Procedure 17. The enzyme employed was 5% hyaluronidase. In all other respects the procedure was carried out as before.

Procedure 18. 5% pepsin in 10 ml 0.7% NaCl was injected into albino mice as a control.

Procedure 19. 1% ptyalin in 10 ml 0.7% NaCl was injected into animals as a control.

Procedure 20. 5% hyaluronidase in 10 ml 0.7% NaCl was injected intraperitoneally.

Procedure 21. Injections of 1% caroid, papain and 1% trypsin, were associated with very high mortality within 48-72 hours.

Procedure 22. 2.5 ml RBC stroma was digested at 37° over night in 7.5 cc 5% pancreatic lipase solution. After repeated shakings the material was filtered and injected into mice as in other procedures.

All mice were sacrificed at 120 hours following injection and smears made from the peritoneal surface as described previously. Slides were stained with Wright-Giemsa and 300-500 white cells counted, except in those instances in which a cellular response was so slight as to limit the observable white cells to 100-200 in 30 minutes of study.

Results. Table I shows that no eosinophile-stimulating substance was dissolved in any lipid solvent. Most of these solvents seemed to destroy or damage the substance. Hot water failed to extract the substance, and boiling for one hour did not add additional amounts. Heat apparently destroyed in large part the eosinophile-stimulating property of stroma. Treatment of red blood cell stroma with alcohol, acetone and alcohol-ether failed to extract

TABLE I. Effects of Solvents, Heat and Enzymes on the Eosinophile Stimulating Substance of Erythrocyte Stroma

Solvents	Animals	% Eosinophiles (mean)	Range
Ether			
Soluble	10	2.6	0.5- 6.3
Insoluble	10	3.52	0 - 8
Petroleum Ether			
Soluble	10	2.23	0 -10.2
Insoluble	7	2.9	0 - 5.6
Chloroform			
Soluble	9	.67	0 - 1.7(43)*
Insoluble	10	2.04	0 - 6.7
Acetone			
Soluble	8	2.31	0 -11
Insoluble	10	10.31	4.7-18.3
Alcohol 95%			
Soluble	9	0	0 - .5
Insoluble	10	7.5	3.7-17
Alcohol—Ether			
Soluble	10	3.32	.4- 6.6
Insoluble	10	10.66	3.6-25.2
N/10 HCl			
Soluble	9	2.3	0 - 7.3(22.3)
Insoluble	10	3.23	.7- 5.7
N/10 NaOH			
Soluble	10	3.87	.7-10.7
Urea 7.5%			
Soluble	10	1.72	.7- 4.5(12)
Hot Water Extraction			
Soluble	9	.9	0 - 2 (12.3)
Insoluble	6	2.08	0 - 5.5(30.6)
Boiling			
Soluble	7	.7	0 - 1 (15.6)
Trypsin 1%			
Soluble	10	0.2	0 - 1.5
Insoluble	10	2.7	0 - 7.3
Pepsin 5%			
Soluble	16	2.8	0 -6.3(16-17)
Insoluble	18	3.9	0 - 9
Caroid 1%			
Soluble	8	2.4	0 - 5.5(15)
Insoluble	12	5.7	0 -12.4
Papain 0.1%			
Soluble	12	5.3	3.6- 8.3
Insoluble	16	5.1	0 -13
Ptyalin 1%			
Soluble	9	5.7	3 - 8.3
Insoluble	9	3.9	1.7- 8.7
Hyaluronidase 5%			
Soluble	9	8.8	0.5-20.3
Insoluble	9	2.2	0 - 5.3
1% Ptyalin	16	11.4	2.3-24
5% Pepsin	5	3	1.3- 6.0
1% Hyaluronidase	10	8.5	2.6-16.6
5% Hyaluronidase	10	9.6	2.0-27.5
Lipase			
Soluble	4	0	0 - 0
Insoluble	10	5.7	2 -11

*Parenthetic figures represent an exceptionally high deviant count found in a single animal and not included in the mean.

a soluble eosinophile-stimulating substance, but did not damage it in the residue. N/10 NaOH caused total solution and partially damaged the substance. N/10 HCl solution resulted in a precipitable fraction at the presumed isoelectric point; both soluble and insoluble fractions showed reduction in activity, the activity being slightly greater in the precipitated portion.

Enzymatic digestion was little more successful than the solvents in producing solution of the E.S.S. of RBC stroma. Trypsin produced nearly complete solution of the entire stroma, but in doing so altered the material so that no further eosinophile stimulus occurred. There was 5% pepsin soluble response, but procedure 17 revealed that pepsin itself produces an eosinophilic reaction. Hyaluronidase digestion produced a solution that provoked eosinophilic response, but control procedure indicates an even more striking response when hyaluronidase alone is used. Papain solution of stroma resulted in a response, but in view of the inability to control this procedure by reason of the early deaths of mice injected with papain alone this observation is not useful.

Some degree of eosinophile stimulating activity was preserved in the insoluble portion after digestion with all protein-splitting enzymes. It was least after tryptic and hyaluronidase and greatest after caroid digestion.

The range of eosinophiles in some of the experiments was rather wide. It was not uncommon to encounter 20-30% eosinophiles in one of 10 animals, when all the others presented 1-5% in their exudate. The significance of this observation will be discussed below.

Discussion. The procedures with solvents indicate that the E.S.S. is not a lipid. Damage to the E.S.S. in the residue by ether, petroleum ether and chloroform suggests that the material may be a protein or a protein-linked carbohydrate. The protection of the substance in the residue by alcohol, by alcohol in combination with ether and by acetone may be expected of either protein or protein-carbohydrate compound.

Destruction of E.S.S. by heat, urea solution, N/10 NaOH, N/10 HCl, and tryptic digestion indicates that at least a part of the substance is protein. The simpler protein fragments of tryptic digestion, as shown in procedure 12, do not evoke an eosinophilic response. Nor, as was reported previously(2), do large protein molecules such as the proteins of plasma or of hemoglobin produce eosinophile migration.

Since weaker proteolytic enzymes of the order of pepsin, caroid and papain fail to eliminate the eosinophilic response, it may be suspected that protein linkage with another type of compound is involved. The procedures reported with fat solvents suggest the linkage is not with a lipid, a point further substantiated by the failure of pancreatic lipase to affect the E.S.S.

There remains the probability that the E.S.S. is a protein-carbohydrate complex. Favoring such a hypothesis is the demonstration that pepsin, ptyalin and hyaluronidase when injected alone produce an eosinophilic reaction. Both ptyalin and pepsin are prepared from biological materials and both are associated with blood group substances that are known to be mucopolysaccharides. Alternately these enzymes and hyaluronidase may release some kind of E.S.S. from the peritoneal surfaces.

It is evident from these procedures that further efforts to fractionate RBC stroma will be unsuccessful since all chemical separations will depend upon obtaining a solution of undamaged E.S.S.

Further work is under way to approach the problem through successive injections of increasingly complex proteins and of increasingly complex carbohydrates as well as to determine if the physical state of material, gel or insoluble suspensions, may determine an eosinophilic response.

The occurrence of an occasionally high eosinophile count in one of 10-20 animals may be the result of an unknown biological difference. In the course of many injections it is almost certain that trauma and peritoneal bleeding will occur from time to time. It is also possible that in spite of efforts to secure a complete mixture of solvents with the gelatinous and gummy stroma, there may not be complete contact between some portions of the mass and the solvent.

Summary. 1. A series of experiments with variously treated RBC stroma injected intraperitoneally into mice clearly demonstrates that an eosinophile-stimulating substance previously noted is not a lipid. 2. The experiments further establish that the substance is either a protein or a protein-carbohydrate compound. 3. The possibility is raised that the physical state of material (gel or insoluble suspension) may determine the eosinophilic response.

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Serum Enzyme Alterations in Cancer. (26999)

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Alterations of serum enzyme levels have been observed in numerous maladies(1,2,3). The fact that similar alterations in level of a particular enzyme such as lactic dehydrogenase occur in many diverse pathological lesions limits the use of a single enzyme as a specific diagnostic tool for cancer detection. The present study was undertaken to determine whether patients with various malignant tumors would reveal different patterns of multiple serum enzyme changes indicative of individual tumor types.

Materials and Methods. The serum enzymes assayed included: lactic dehydrogenase (LDH), glutamic pyruvic transaminase (SGPT), glutamic oxaloacetic transaminase (SGOT), malic dehydrogenase (MDH), aldolase (ALD), enolase (ENOL), pyruvic kinase (PK), myokinase

TABLE I. Serum Enzyme Activities[1] in Normal Controls and Patients with Malignant and Non-Malignant Diseases.

Enzyme	Normal Controls	Hernias	Benign Tumors	Cholecystitis	Cancer of Female Reproductive system	Cancer of Stomach	Cancer of Intestines
	N	N	N	N	N	N	N
LDH	26	10	14	11	9	12	49
	189 ± 13.3	236 ± 14.4*	196 ± 17.0	306 ± 38.0*	615 ± 151.9*	312 ± 513*	336 ± 30*
SGPT	26	10	14	11	10	12	49
	17 ± 1.0	14 ± 1.7	14 ± 1.9	27 ± 9.1	14 ± 1.8	11 ± 1.0*	24 ± 5.6
SGOT	26	10	14	11	10	12	49
	15 ± .9	15 ± 1.7	15 ± 1.9	25 ± 5.1	19 ± 3.4	13 ± 1.1	23 ± 4.3
MDH	26	10	14	11	9	12	49
	148 ± 13.9	169 ± 26.0	168 ± 21.6	201 ± 50.4	403 ± 69.5*	227 ± 39.0	281 ± 24.9*
ALD	25	10	14	10	9	11	48
	4 ± .6	1 ± .4*	4 ± .7	4 ± 1.3	6 ± 1.3	3 ± 1.4	5 ± 1.2
ENOL	26	10	14	11	10	12	49
	9 ± 1.0	7 ± .8	11 ± 1.7	12 ± 2.9	26 ± 6.9*	12 ± 1.6	17 ± 2.0*
PK	26	10	14	11	10	12	49
	15 ± 1.2	9 ± .7*	13 ± 2.0	10 ± 2.4	18 ± 5.7	16 ± 4.9	11 ± .8*
MK	26	10	14	11	10	12	47
	9 ± 1.3	6 ± .6*	9 ± .7	10 ± 2.2	12 ± 2.2	6 ± 1.5	11 ± 1.2
PGK	24	10	13	11	9	12	42
	9 ± 1.2	8 ± 1.0	13 ± 2.5	10 ± 2.8	14 ± 2.6	13 ± 1.7	10 ± 1.5

Values shown are means together with standard errors.

[1] One unit of activity = change in density of 0.001/ml of serum/min at 24-26°C.

* The difference in means as compared with normal controls is statistically significant to the 0.05 level or more using Fisher's "t" test for significance.

(MK), phosphoglyceryl kinase (PGK), glyceraldehyde phosphate dehydrogenase (GAPDH), hexokinase (HK), creatine kinase (CK), and a combined α-glycerophosphate dehydrogenase-triose phosphate isomerase (GDH/TIM).

The methods employed for each assay have been described previously(4).

Sera were obtained from patients with various malignant lesions with metastases, non-malignant ailments requiring surgery, and from healthy hospital personnel and blood donors who served as normal controls. The age distribution in the non-cancer groups (controls consisting of normal hospital personnel, patients with hernia, cholelithiasis without infection and benign tumors) is similar to that of the cancer groups (stomach, intestine and female reproductive tract). We have also established that for at least one of the enzymes (LDH), there is no correlation between the serum level of this enzyme and age or sex. Enzyme assays were completed within 24-30 hours after collection of the blood; hemolyzed sera were rejected. Clinical diagnoses were confirmed by pathological examination of the tissue removed at operation. Sera were run independently of the diagnosis, and were correlated with diagnoses only after calculation of enzyme activities.

Results. In the initial 70 sera examined, HK, CK, GAPDH, and GDH/TIM showed little or no measurable activity in the sera of normal and diseased patients and these enzymes were consequently dropped from further study.

Preliminary results reported earlier indicated that statistically significant elevations of LDH, MDH, and ENOL activity occurred in groups of patients with cholecystitis, cancer of the intestine, cancer of the stomach, and cancer of female reproductive organs(5,6). In this preliminary group elevations of MK and PGK occurred in cancer of the intestine and cancer of female reproductive organs. ALD was elevated in cancer of the female reproductive organs, SGPT in cancer of the intestine, SGOT in the latter 2 groups and in the group with cholecystitis. PK levels were found to be significantly lower in the cholecystitis group. Patients with benign tumors showed no significant enzyme alterations when compared to the normal controls.

Subsequently, studies were carried out on a larger group of patients, who had not received prior therapy of any kind. The data obtained from these screened groups of patients are summarized in Table I. Significant elevations of LDH occur in all of the groups except those with benign tumors. Elevations of MDH and ENOL occur most frequently in the groups with cancers of the intestine and the female reproductive organs. Significant lowering of activity of ALD, PK, and MK occurred in patients with hernias. Similarly, SGPT levels in patients with cancer of the stomach, and PK levels in patients with cancer of the intestine were found to be significantly lower. Table II summarizes the observed ranges of LDH activity of the several groups studied. Variations in similar proportions were found in most of the enzymes studied. For example, in the group of patients with cholecystitis a range of 8-104 units of SGPT activity, with a mean of 27 and a median of 17, was observed.

TABLE II. Observed Ranges of LDH Activity* in Normal Controls and Patients with Malignant and Non-Malignant Pathologies.

Group	N	Observed Range	Median	Mean
Normal Controls	26	63-300	210	189
Hernia	10	165-300	250	236
Benign Tumors	14	100-308	187	196
Cholecystitis	11	145-575	290	306
Cancer of Female Reproductive Organs	9	220-1400	400	615
Cancer of Stomach	12	150-700	267	312
“ “ Intestine	49	90-1050	268	336

* 1 unit of activity = △ O. D./ml of serum/min. at 24-26°C.

Discussion. This study is not one of individual cancer patients, but rather an attempt to ascertain whether a *group* of patients with a particular tumor type has a pattern of enzyme change which is *characteristic* of the group. All of the patients in the various cancer groups had metastases, some involving the liver. The effect of specific metastases, particularly to the liver is being assessed currently. However, in the group as a whole such metastases did not appear to influence the enzyme pattern.

Patterns of alteration indicated in Table I are applicable to the group and do not necessarily reflect the finding in each individual within the group. Table II serves to highlight the fact that there is considerable variation within each group, including the normal controls. However, repeated determinations reveal a constant level in the individual controls. Among pathological groups, elevations of LDH were frequently associated with elevations of MDH, however, elevations of other enzymes occurred in an apparently random fashion.

The activities of ALD, ENOL, PK, MK, and PGK ranged upward from zero in all groups studied. Thus, it is impossible to differentiate between individuals from a group whose mean value for one of these enzymes was significantly lower from individuals whose enzyme levels are normally low.

The significance of variation in individual enzyme levels becomes apparent only when serial determinations are used as a parameter. The statistical evaluation of the data in this study indicates that a determination of enzyme level in a single sample of serum may be misleading. For example, a rise from 70 to 275 units of LD in an individual is significant even though both levels may be considered to fall within the normal range. While the values in a group of individuals might encompass a large range, the determination for any one member of the group might show little variation in contrast to that manifested by the group. This is demonstrated by the data in Table III, showing a group of normal subjects whose serum levels were repeatedly determined at different times, in some instances over a period of more than a year. From this, it is apparent that with almost all of the enzymes, each individual control tends to retain a value similar to that shown earlier.

In view of the above it would be profitable

TABLE III. Variation in Serum Enzyme Activity of Normal Controls.

			LDH	SGPT	SGOT	MDH	ALD	ENOL	PK	MK	PGK
A	obese	5/5/59	330	22	24	160	6	8	29	5	10
		12/20/60	300	26	30	200	4	11	6	6	10
B		10/10/60	135	8	11	150	0	2	2	4	4
		1/3/61	125	9	13	150	0	9	10	11	2
C		11/21/60	125	25	14	125	0	12	10	14	6
		1/3/61	110	27	14	125	1	6	6	11	4
D		10/3/60	225	17	16	200	2	8	7	6	4
		12/15/60	175	22	14	175	3	7	6	7	4
E		11/14/60	125	16	10	100	0	8	9	7	5
		1/3/61	115	16	14	100	2	6	6	10	4
F		3/4/60	175	9	10	110	4	7	9	7	5
		3/14/60	180	8	10	100	0	5	8	14	5
G	Negro	11/30/59	100	17	14	75	5	5	16	3	5
		12/19/60	85	20	16	110	0	2	6	4	4

to obtain serial determination at different times to establish the base levels for the individual. These individuals can then be followed at regular intervals and should changes occur, search could be instituted for a basic lesion responsible for this alteration. Studies in this direction have been initiated using a group of healthy hospital personnel and individuals examined at the Cancer Detection Clinic at this hospital.

Summary. Data have been presented on alterations in serum enzyme activity in patients with malignant and non-malignant diseases as compared to normal controls. Patterns of change have been established for the various groups. The patterns for the malignant groups differ statistically from those of the non-malignant ones. The malignant groups differ from each other.

Every individual in each group does not show all the changes described for the group. There is considerable overlap between pathological and control values. These discrepancies are discussed in terms of what is meant by "normal" range. Data are presented on the "normal" range of controls.

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Histochemical Evidence of Aldehyde Blocking Capacity of Lathyrogenic Agents. (27000)

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On the assumption that aldehyde blocking agents might produce lathyrism, Levy(1) grew salamander and toad tadpoles in water containing various aldehyde blocking agents. The greater number of those agents were found to produce tumors of the notochord. In this study an attempt has been made to determine the ability of these lathyrogenic agents to block

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[*] National Science Undergraduate Research participant.

TABLE I. Aldehyde blocking capacity and lowest effective dosage for lathyrogenesis of certain compounds.

Chemical	Molarity						Lowest concentration effective in producing tumors of notochord (Levy).	
	0	.00001	.0001	.001	.01	.1	mg/100 ml	Molarity
Phenylhydrazine	++++	++++	+	0	0	0	1	.00009
1-Methyl-1-phenylhydrazine	++++	++++	+++	+	0	0	.5	.00004
4-Phenyl-3-thiosemicarbazide	++++	++++	+++	++	−		3	.00018
Thiosemicarbazide	++++	++++	+++−	+++	+	0	10	.00110
1-Benzyl-1-phenylhydrazine HCl	++++	++++	++++	+++	+	0	1	.00004
Semicarbazide HCl	++++	++++	+++−	+++	++	+	1	.00013
BAPN	++++	++++	++++	+++	++	+−	1	.00014
Amino antipyrine HCl	++++	++++	++++	++++	++++	+++−	50	.00208
Urea	++++	++++	++++	++++	++++	++++	1	.00017
AAN	++++	++++	++++	++++	++++	++++	1	.00017

aldehydes in a histochemical test and to compare this ability with their capacity to produce lathyrism in tadpoles.

Materials and methods. Liver tissue, selected for its high glycogen content, was obtained from 4 rats, 2 guinea pigs and 5 rabbits. All were well-fed normal animals. Thin slices of liver were fixed in cold alcohol-formalin or quenched in liquid propane at −170°C and stored in acetone at −70°C for 10 days. After fixation, all tissues were embedded in paraffin. Six micron thick sections of the liver of each animal were dry mounted on slides, deparaffinized, hydrated and oxidized with 0.5% periodic acid in 70% alcohol for 10 minutes. The sections were then incubated for 1 hour at 37°C in distilled water containing serial dilutions (starting with 0.1 molar) of the following lathyrogenic agents: amino antipyrine HCl, phenylhydrazine HCl, semicarbazide HCl, urea, aminoacetonitrile hydrogen sulfate (AAN)†, and beta-aminopropionitrile (BAPN). Because of low solubilities, serial dilutions of a saturated solution of 4-phenyl-3-thiosemicarbazide were used while 1-benzyl-1-phenylhydrazine HCl was dissolved in 70% alcohol. Control sections of each liver were incubated in water or 70% alcohol. As in Levy's experiment, the pH was not adjusted. After incubation all slides were rinsed with distilled water, stained for 30 minutes in Schiff's reagent, rinsed in sulfurous acid, washed in running tap water for 10 minutes, dehydrated, cleared, and mounted in Permount. The staining reaction for each slide was then graded using 4+ when full staining was present and zero when total blocking of staining had occurred.

Results. The results are shown in Table I where the compounds tested have been arranged in order of their capacity to block the histochemical aldehyde-Schiff reaction. The table also shows the minimum concentration of the compounds shown by Levy to be effective in producing tumors of the notochord, and a) that the compounds differ widely in their ability to block aldehydes and b) their ability to block aldehydes *in vitro* differs from their ability to produce tumors of the notochord in tadpoles.

Discussion. Although an agreement of the results of this study and the study of Levy would have been good supporting evidence that the mechanism of lathyrogenesis is aldehyde blocking, no such agreement exists. However, the differences observed in the 2 experiments may be due to inherent differences between *in vitro* and *in vivo* studies. Because urea(2) and AAN showed no aldehyde blocking capacity, this study suggests that the lathyrogenic compounds may have some other characteristic in common which may be responsible for production of lathyrism in tadpoles.

Summary. This study has demonstrated that compounds known to possess lathyrogenic properties may, or may not, block the periodic acid-Schiff reaction, and that for these compounds, no correlation exists between their aldehyde blocking capacity and their ability to produce lathyrism in tadpoles. The mechanism by which those compounds produce lathyrism therefore continues to be obscure.

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† Generously furnished by Abbott Laboratories, North Chicago, Ill.

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"Epithelial Transformation" of Human Synovial Connective Tissue Cells: Cytologic and Biochemical Consequences. (27001)

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While it is generally recognized that cells cultured *in vitro* may undergo "alteration" during the course of serial propagation, the precise nature of the observed changes remains obscure. One distinctive pattern of alteration is well illustrated by the 8 Detroit strains of human epithelial-like cells(1). In these cell lines, cultures of fibroblast-like cells derived from bone marrow, pleural and ascitic fluid, developed plaques of cells characterized by an epithelial growth pattern, rapid multiplication, giant nucleoli, and polyploidy. Since cell lines which accomplish this "transformation" are sometimes capable of extended propagation *in vitro*, they provide a system which can be studied on a mass scale over a prolonged interval. Under these circumstances, the cellular system might be used to study "retained" structural and biochemical characteristics on the one hand, while on the other hand this system may unfold for critical scrutiny the biochemical alterations and related changes in morphology that provide the new cell with an improved outlook for survival in the abnormal environment of the culture flask.

The present report describes the cytologic and biochemical consequences of a fortuitous "epithelial transformation" of a cell population derived from human synovial tissue. In previous investigations, microscopic examination of primary outgrowth from normal and malignant synovial tissue revealed cells with fibroblast-like morphology(2,3), while functional studies have demonstrated the capacity of these cells to form mucopolysaccharides(4,5,6,7,8,9). In view of the biochemical changes documented in this communication, one might hope that understanding of such "transformations" would be advanced by systematic study of biochemical parameters in addition to the usual microscopic observations.

Materials and Methods. Synovial tissue was obtained by needle biopsy from the knee-joint of a 50 year-old man with rheumatoid arthritis of 6 months duration. This diagnosis was supported by clinical examination, x-ray evidence, a Latex fixation test for rheumatoid factor positive in a titer of 1/5120, and by the pathologic interpretation of the biopsy itself. At another hospital the patient had had a 10-day course of treatment with triethylenemelamine. Explantation of tissue fragments was carried out in T-flask under perforated cellophane as described earlier(9). Establishment of monolayer cultures, feeding schedules, and harvesting techniques were carried out as in previous studies(9).

Cell enumeration and volume measurements were carried out with a model B Coulter Electronic Cell Counter and Cell Size Analyzer. Hyaluronic acid was measured in culture medium as uronic acid by the method of Bollet as well as by a method developed in this laboratory(10,11). Hexose was determined by

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the anthrone method(12), while cellular protein was estimated by a modification of the procedure described by Lowry(13). RNA and DNA were measured by the orcinol and diphenylamine reactions for their respective carbohydrate moieties(14). These latter 2 analyses were carried out on the cellular fraction extracted by 10% trichloroacetic acid at 90°C in 15 minutes. In an effort to detect the presence of the oligosaccharides of hyaluronic acid in culture medium, ethanol fractionated medium was subjected to the charcoal column procedure described by Rapport, Meyer and Linker(15).

Media used in this study included CMRL 1066(16), Eagle's basal medium(17), and "Yela medium." The latter is an undefined medium consisting of a mixture of 0.1% yeast extract and 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution. Experiments with agitated suspension cultures were carried out on a New Brunswick Model G10 rotary shaker at speeds of 120-130 rpm. Cytologic studies on cover-slip preparations were performed with the aid of the May-Grunwald Giemsa and Wright's stains. Chromosome enumeration was carried out on acetic orcein stained specimens prepared by the method of Tjio and Puck(18).

Results. History of culture through period of "transformation": Biopsy fragments were explanted in a T-15 flask under perforated cellophane and nourished by a medium containing 80% CMRL 1066 and 20% human serum, supplemented with L-glutamine (2mM), penicillin (200 μg/ml) and streptomycin (200 μg/ml). Radial outgrowth appeared in 3 days and was sufficient in 17 days to permit trypsin dispersion and subculturing as a monolayer culture. The culture grew relatively rapidly with an average culture generation time varying between 36.5 hours and 80.2 hours. At the second subculture the supernatant medium gave a positive mucin clot test with acetic acid, (0.13 ml 7.0 N acetic acid added to 5.0 ml culture medium). The mucin clot test on culture medium was variable during the first 3 months *in vitro*, and eventually disappeared altogether. About 85 days after explantation, at the tenth subculture, the culture growth rate slowed strikingly and generation time increased to the range of 80 to 130 hours. During this phase of slow growth, epithelial-like plaques appeared in numerous places in the culture vessel. These epithelial-like cells rapidly overgrew the ordinary fibroblastic cells, and soon became the representative cell form. Once this "transformation" had taken place, the cells returned to their previous rate of growth, although permanently losing the capacity to induce mucin clot formation in the supernatant medium.

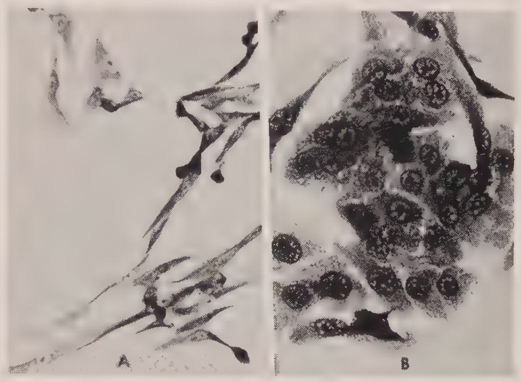

FIG. 1. Photomicrographs of synovial cells before and after transformation. X65. (A) Spindle-shaped cells prior to transformation, (B) polygonal cells after transformation. (Relative measurements on original preparations confirm equality of magnification of (A) and (B).)

Cytology. Stained cover slip preparations illustrate the striking alteration in morphology (Fig. 1). The pre-transformation fibroblastic cells are spindle-shaped and grow in a reticular pattern, in contrast to the post-transformation colonies of polygonal cells growing with contiguous cell borders. The photomicrographs, made at the same magnification, demonstrate the nuclear and nucleolar enlargement in the transformed cells. The nucleolar diameter of the transformed cells is frequently 3 times that seen in the unaltered cells.

Chromosome counts prior to the "transformation" indicated that the normal human complement was numerically dominant in the cultures. On the other hand, the post-transformation cultures revealed a markedly abnormal chromosome make-up, with virtually no cells showing the normal number of chromosomes. The magnitude of this shift in chromosomal complement is revealed in Table 1.

Culture behavior. Although the epithelial shape and growth pattern first drew attention

TABLE I. Exaggerated heteroploidy following "epithelial transformation."

"C. W." Rheumatoid Synovium	NO. OF CHROMOSOMES PER CELL					
	40-45	46	47-80	80-90	"Near Tetraploid" 91-94	100+
Pre-transformation 3rd Subculture (28 Cells Counted)	35%	61%	—	4%	—	—
Pre-transformation 5th Subculture (9 Cells Counted)	56%	44%	—	—	—	—
Post-transformation 13th Subculture (25 Cells Counted)		4%	24%	8%	40%	24%

to the cellular alteration, several other peculiarities of the new cell were soon evident. The transformed cells had a reduced affinity for glass surfaces and frequently a newly inoculated suspension of cells required 2 or more days to attach to the surface of a "T-flask." This reduced "adhesiveness" of cells for glass was in large part corrected by incorporating 5 to 10% fetal calf serum in the nutrient medium, and to a lesser extent by treating the "T-flasks" with 0.2 N NaOH prior to final rinsing and sterilization. The transformed cell cultures rapidly acidified the medium, occasionally requiring neutralization twice daily. When the medium pH fell to approximately 7.0, a large portion of the cells detached from the glass and floated freely in the supernatant medium. Cells could be promptly removed from a glass surface with 0.25% trypsin, and in addition by 0.01 M EDTA. Limited previous experience indicated that EDTA was an ineffective agent for separating unaltered primary synovial cultures from glass.

While previous experience with primary synovial culture suggested that synthetic medium CMRL 1066 was superior to Eagle's basal medium, the transformed cells thrived in flask cultures nourished by 80% Eagle's basal medium and 20% human serum. Further, they grew reasonably well in an undefined medium where 20% serum was supplemented by 80% Yela medium.

While previous attempts to grow unaltered synovial cells in agitated suspension culture have failed, in our hands, to realize a net increase in cell numbers, the transformed cells were capable of moderate growth under these circumstances. In one experiment with agitated suspension cultures, 1.3×10^7 cells were suspended in 108 ml of culture medium composed of 80 ml Yela medium, 20 ml human serum, 1.0 ml 200 mM L-glutamine, 1.0 ml 100 × penicillin-streptomycin solution, 2.0 ml 0.05 M EDTA, and 4.0 ml 4% methylcellulose. The cell suspension was placed in a 250 ml Erlenmeyer flask and agitated at 130 rpm on a rotary shaker in a 37°C incubator room. At intervals during the 16-day culture period, 1.0 ml volumes of 100 × glucose, amino acids*, and vitamin stock solutions* were added (Fig. 2).

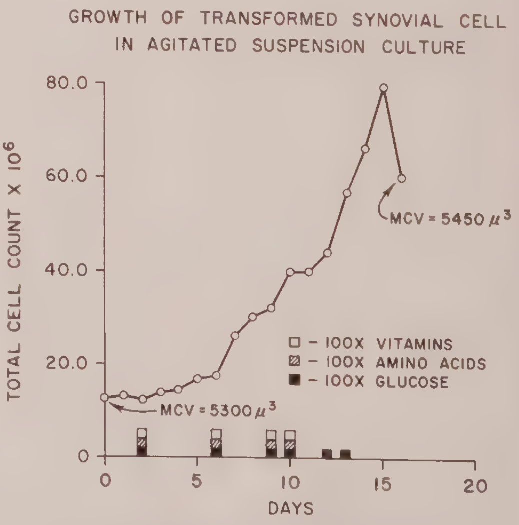

FIG. 2 A 5-fold increase in population occurred during the second week of agitated culture.

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* These stock solutions are those used in preparing Eagle's basal medium.

TABLE II. Comparison of chemical parameters of "transformed" cells with those of "normal" and "malignant" cells.

PARAMETER MEASURED	"Normal" Synovial Cells		"C.W." Cells Pre-Transformation		"C.W." Cells Post-Transformation		J-111 "Malignant Monocytes"	
	No. Obs.	Mean ± S.E.*	No. Obs.	Mean ± S.E.	No. Obs.	Mean ± S.E.	No. Obs.	Mean ± S.E.
Mean Cell Vol-μ^3	90	7421 ± 265	—	—	16	5669 ± 248	9	4987 ± 289
Cell Protein, 10^{-12} g/cell	50	380 ± 19	—	—	9	1070 ± 75	7	794 ± 65
μ g DNA/mg Cell Protein	4	32 ± 6.6	4	40.0 ± 8.5	4	40.0 ± 3.2	7	58 ± 4
RNA/DNA	4	3.33 ± .71	4	3.43 ± .70	4	1.32 ± .48	7	1.40 ± .22
Hexose Uptake mg/mg cell protein/day	33	4.04 ± .49	—	—	7	1.26 ± .07	5	1.62 ± .23
Hyaluronic Acid Production 10^{-12} g/cell/day	76	19.6 ± 1.8	1	29.6	5	1.64 ± .59	—	—

* Standard error of mean

A 5-fold increase in cell numbers took place after a prolonged lag period (Fig. 2). Non-dialyzable acid mucopolysaccharide was present in the medium in concentrations of 3.7 μg/ml, (calculated as hyaluronic acid) a value 2 to 6 times greater than that found in static cultures. Calculation of the comparative rates of synthesis, taking into account the prolonged contact of cells with the medium in the agitated cultures, indicate that the cells in the suspension culture form hyaluronate at a rate similar to that found in static cultures.

Structural and functional characteristics. Data concerning the size, protein, and nucleic acid content, as well as hexose consumption and hyaluronate synthesis of the altered cell line (grown in static cultures) are summarized in Table 2. The transformed cell, although slightly smaller than the average "normal" synovial cell, appeared to contain over twice as much protein. Prior to "transformation" the cells in question exhibited DNA/protein and RNA/DNA ratios which closely resembled the values commonly seen in unaltered cells derived from human synovium. Following the alteration in morphology, the RNA/DNA ratio decreased, without evidence of change in the DNA/protein ratio. The quantity of RNA per cell appears to be unchanged in the face of over 2-fold increases in cellular protein and DNA.

Measurements of hexose uptake indicated that the transformed cell removed glucose from the medium at less than one-half the rate common to healthy proliferating synovial derivatives, when cell protein measurements were used as a basis for comparing rates. The decreased rate of hexose uptake (per unit of cell protein) is nearly the reciprocal of the increase in protein per cell, suggesting that the actual uptake of glucose per cell is virtually the same in the normal and transformed cells.

Hyaluronic acid synthesis fell off drastically following the transformation. Loss of the positive mucin clot reaction in the culture medium, as well as a profound reduction in chemically measurable uronic acid-containing acid mucopolysaccharide, both point to decreased elaboration of high molecular weight hyaluronate. Since the procedures used above do not distinguish between defective polymerization of hyaluronate and decreased elaboration of the disaccharide repeating unit, (or larger oligosaccharides), we undertook the identification of oligosaccharides in used culture medium. No trace of uronic acid-containing material was found in the medium from transformed cultures, although disaccharide and tetrasaccharide added to unused medium could be recovered in the pyridine eluate from a charcoal column.

Discussion. The dramatic appearance of colonies composed of epithelial-like cells in the midst of fibroblast cultures quite properly raises the question of contamination by one of the established epithelial cell lines. Fortunately, we have not carried epithelial cell lines and the only stable cell line of non-synovial origin present in the laboratory at time of the transformation was the J-111 line derived from the peripheral blood of a woman with acute monocytic leukemia(19). Morphologic and chemical data do not support the possibility that the transformed cells were contaminants from the monocyte line. It is unlikely that skin epithelium was carried into the original biopsy material, since the procedure is carried out through a trocar after a skin incision. Available cytologic and chemical data suggest that the pre-transformation cell was similar to material examined in our previous experience with normal synovial connective tissue cell derivatives. If one uses the cellular protein values from the collected normal synovial cultures as a base line, the major compositional changes in the transformed cell would appear to be 2-fold increases in cellular protein and DNA.

While the data do not suggest a quantitative alteration in cellular RNA, a qualitative change would not be detected by the methods employed. Cytologic measurements of nucleoli, the locus of nuclear RNA, indicate that the volume of this structure increased 8-fold or more in the transformed cell, lending some support to the notion that at least a shift in the proportions of the various RNA fractions may have taken place. Such an alteration in the enzyme forming systems of the cell provides a reasonable explanation for the decreased hyaluronate synthesis observed. The absence of significant amounts of oligosaccharide in the medium suggests that the decreased synthesis of high molecular weight hyaluronate largely resulted from reduced synthesis of the low molecular weight repeating units, rather than failure of polymerization.

Changes in the form and growth pattern exhibited by the transformed cell may reflect alteration in the character of the plasma membrane. Acquired resistance to the battering effects of agitated suspension culture could be explained in the same way. Decreased adhesive properties of the cell as manifested by increased ease of separation from glass with trypsin, versene, or fall in medium pH, all suggest that the negative charge density of the plasma membrane was reduced. While these changes in culture behavior may all be construed to reflect some modification of the plasma membrane, there is no reason to believe that a single change in the chemical structure of the membrane is responsible for the diverse effects.

Summary. Primary cultures of fibroblast-like cells from rheumatoid synovial tissue changed *in vitro* to a cell population exhibiting epithelial morphology, heteroploidy, altered growth characteristics, and decreased capacity to synthesize hyaluronic acid. Quantitative changes in the protein and nucleic acid composition of the cells were also demonstrated.

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Connective Tissue V. Effect of Topical Trypsin upon Dermal Chemical Response to Local Inflammation and Necrosis.*‡ (27002)

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Interest in the enzymatic debridement of wounds reaches back to John Hunter, who is reported to have employed pancreatic juice for this purpose(1). Later, Morris claimed that pancreatic extracts would clear the eschar of a burn within 4 hours(2).

Connell and Rousselot(3) reported that trypsin was useful as a debriding agent, and Wilde and Derry(4) indicated that trypsin was of use in debridement of burns. Reiser *et al* have discussed in detail the tryptic debridement of necrotic tissue(5,6).

Baetzner's original description of the antiphlogistic action of parenteral trypsin(7) has been developed by Innerfield(8) on the basis of the destruction of the proteinaceous barriers to capillary permeability, and recent experimental work(9) has shown that injection of trypsin into bruised tissue increased the rate of tissue repair.

This paper describes the chemistry of the response of rat skin to local croton-oil induced inflammation, and compares these results with the chemistry of the inflammatory response in lesions which have been topically treated with trypsin.

Materials and Methods. One hundred and twenty male Sprague-Dawley rats (300-330g) were subjected to intradermal injection, into a previously shaven area of abdominal skin (right side), of 0.4ml of a dilution of the chemical irritant croton oil made up to a concentration of 75% irritant in an inert carrier of peanut oil. After eschar formation (3 days), the resulting necrotic lesions of one group of animals were sprayed topically with a solution containing 1 mg/ml of crystalline trypsin in isotonic saline daily for 20 days. The other group was subjected to daily topical applications of saline alone. Separate control studies indicated that the daily topical application of trypsin to shaven areas of intact rat skin was without effect upon the dermal chemistry while the application of saline alone to the necrotic lesion did not result in significant alterations in the patterns of the chemical response of the skin to similar concentrations of croton oil.

At various times after injury, 8 animals from each group were sacrificed by etherization and exsanguination and both the necrotic lesion and about 1.5 g of uninjured abdominal skin distal from the site of injury were removed, dissected free of adhering hair, fat, fascia and muscle, weighed and stored in the frozen state. Tissue samples obtained before and after eschar sloughing were lyophilized, and their water content determined by weight loss.

The necrotic lesions, and aliquots of the uninjured skin from each animal, were hydrolysed for 8 hours at 100°C in 10ml/g of 4N HCl. Triplicate samples of these hydrolysates were removed and analysed in triplicate for nitrogen(10), hydroxyproline(11), and hexosamine(12). These results were translated via a standard curve into μmoles or mmoles per g of fresh, wet, cleaned rat skin. The mean and standard deviation of the 9 determinations for each analysis are reported in the tables.

The cleaned and shaven uninjured abdominal skin remaining (ca. 1.0 g) was pooled into 2 groups of 4 animals each, and 3.5 g of skin from each pooled sample of tissue was extracted sequentially in the cold with isotonic saline, 0.5M NaCl and 0.5M citrate buffer (pH 3.6) as has been described previously(10,13). Triplicate samples of each extract after centrifugation were similarly hydrolysed, along with one ml of sera from each animal, in 4N HCl, and their nitrogen, hydroxyproline and hexosamine content determined. The mean and standard deviation of each set of determinations are presented below. These extracts have been shown previously to extract quantitatively the ground substance, neutral soluble and acid soluble collagens respectively(13).

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Experimental and Results. Grossly, the croton oil injury produced an ulcerous lesion which was lightly outlined by erythema. After 3 days, both the trypsin treated and the control groups of animals formed an eschar within a period of 24 hours. Isolation and contraction of the eschar to the point of sloughing occurred after 13 days in the untreated control group and after 10 days in the topically trypsin treated animals. Thirteen days after sloughing, the tissue remained pink, soft and hairless in the trypsin treated animals, while the skin of the injured area in the control group had become re-covered with hair, and although it still tore easily, was not noticeably different from normal. Mean water content determined by lyophilization and standard deviation of the trypsin treated lesion 2, 4, and 14 days after injury was 65 ± 2, 60 ± 1 and 58 ± 1% respectively, while for the untreated lesion it was 60 ± 1, 58 ± 1 and 55 ± 1% for the same days after injury. Uninjured tissue showed no changes in water content.

Histologically, both injuries showed a typical hemorrhagic pattern of ulceration and necrosis throughout both layers of the skin, resulting in a non-suppurating defect. The erythema was somewhat more noticeable in the trypsin treated lesions, and edema appeared slightly more profound than in the untreated lesions. A few days after eschar isolation, granulation tissue formed in both groups of rats. The granulation tissue was apparently maintained in the trypsin treated group, whereas the control lesion proceeded to heal via fibroplasia and epithelization. The area of skin distant from the local lesion demonstrated no significant histochemical change over the whole time course of the experiment in either group of animals.

The alterations with time after injury of the total dermal concentrations of hydroxyproline and hexosamine at site of croton oil injection with and without daily topical application of trypsin are compared in Table I with the analysis of skin from uninjured animals of similar body weight. These results indicate that there was a marked increase in the hydroxyproline and an increase in hexosamine concentration of the necrotic lesion. Nitrogen concentration of these lesions demonstrated no significant alteration with inflammation. These changes in dermal chemistry were prolonged when the wound was treated topically with trypsin. The application of this enzyme also decreased quantitatively the increment in the dermal concentration of hexosamine which occurred normally in response to local injury.

TABLE I. Hydroxyproline and hexosamine content (μmoles/g skin) of dermal necrotic lesions with and without topical trypsin treatment‡.

Days after Injury	Hydroxyproline (μmoles/g)		Hexosamine (μmoles/g)	
	control	trypsin	control	trypsin
0	240 ± 12	230 ± 12	9.3 ± .5	10.0 ± .5
2	*140 ± 7	*133 ± 7	*12.6 ± .6	*12.6 ± .6
3†	*152 ± 7	*126 ± 6	*15.3 ± .7	*12.8 ± .6
4	*180 ± 9	—	*17.3 ± .9	—
5	*100 ± 5	*127 ± 6	*24.0 ± 1.2	*17.3 ± .9
7	*100 ± 5	*105 ± 5	*23.5 ± 1.2	*14.0 ± .7
10	—	*125 ± 6	—	*15.3 ± .7
14	*113 ± 6	*115 ± 6	*21.3 ± 1.1	*18.6 ± .9
23	200 ± 20	*113 ± 5	10.6 ± .5	*14.0 ± .7

† First day of trypsin treatment.

‡ Dermal nitrogen content of both groups of lesions for all days fell within the range of 4.6 to 5.4mmoles/g and was not significantly different from control mean (day 0).

* Significantly different from control mean ($p < .05$).

__ Underlined mean differs significantly from that of untreated lesion ($p < .05$).

Concentration of hexosamine in the wound was unaffected by formation of the eschar in the untreated animals, while the amino sugar content of the trypsin treated wound did not change until the 5th day after injury.

The hexosamine, nitrogen and hydroxyproline concentrations in the sera from rats with croton oil induced dermal injuries with and without topical trypsin treatment are presented in Table II. The circulating concentration of nitrogen or hexosamine was not significantly affected by production of a dermal lesion. Elevations of both materials were found in the sera of those animals whose lesions were treated with trypsin. The results also suggested that topical trypsin treatment of the dermal lesions permits the hydroxyproline containing materials from the necrotic lesion to drain into the circulation.

The uninjured skin from rats distal to the site of local injury demonstrated a significant decrease in total dermal hydroxyproline con-

TABLE II. Effects of dermal necrosis upon analysis of sera of rats with and without trypsin applied topically to the lesion.

Days after Injury	Nitrogen (mmoles/ml)		Hydroxyproline (μmoles/ml)		Hexosamine (μmoles/ml)	
	control	trypsin	control	trypsin	control	trypsin
0	.65 ± .04	.60 ± .04	.36	.36	4.5	4.5
2	.71	*.71	.35	.35	4.5	4.5
3†	.68	*.79	.40	.34	4.3	*14.6
4	.63	—	.40	—	4.5	—
5	.67	*.74	*.48	*.64	4.8	*10.6
7	.63	*.79	*.50	*.52	4.0	* 9.1
10	—	*.81	—	*.73	—	* 7.9
14	.60	*.79	.42	*.58	4.1	* 9.1
23	.63	*.84	.40	.36	4.2	* 8.2

† First day of trypsin treatment.

* Significantly different ($p < .05$) from control mean (day 0).

__ Underlined mean differs significantly from that of untreated lesion ($p < .05$)

TABLE III. Hydroxyproline content (μmoles/g) of rat skin distant from total site of croton oil injury with and without topical trypsin treatment.

Days after Injury	Without Trypsin	With Trypsin
0	240 ± 12	240 ± 12
2	*170 ± 8	*170 ± 8
3†	*176 ± 9	*202 ± 10
4	*173 ± 8	—
5	*171 ± 8	*202 ± 10
7	*173 ± 8	*204 ± 10
10	—	*214 ± 11
14	*203 ± 10	*202 ± 10
23	240 ± 14	*200 ± 10

† First day of trypsin treatment.

* Significantly different ($p < .05$) from control mean (day 0).

__ Underlined mean differs significantly from that of untreated lesion.

centration (Table III). These changes in hydroxyproline were not associated with significant alterations in the dermal concentration of either hexosamine or nitrogen, however. Trypsin treatment of the local lesion both prolonged this "distal dermal collagen response" to local inflammation and decreased these losses of hydroxyproline from the uninjured skin.

The uninjured skin distant from the site of local injury was extracted sequentially in the cold as described previously. The hydroxyproline content of the 0.15 and 0.50M saline extracts obtained from the uninjured skin of the control group of animals is compared with the results using the skin from trypsin treated animals in Table IV. These results indicate that the isotonic saline soluble imino acid content of uninjured tissue was not affected by either local inflammation or topical trypsin treatment of the local lesion. The 0.5M saline soluble hydroxyproline concentration of these tissues decreased with distant local injury, however, and this decrease was statistically less profound when the local lesion was sprayed with trypsin.

TABLE IV. Saline soluble hydroxyproline concentration (μmoles/g) of rat skin distant from site of croton oil injury with and without topical trypsin treatment.

Days after Injury	Extract .15M NaCl		Extract .50M NaCl	
	control	trypsin	control	trypsin
0	4.6 ± .5	4.5 ± .5	19.3 ± 1	19.0 ± 1
2	4.4 ± .5	4.8 ± .5	* 9.3 ± .5	* 8.8 ± .4
3†	4.5 ± .5	4.7 ± .5	* 9.0 ± .5	*10.6 ± .5
4	4.4 ± .5	—	* 9.3 ± .5	—
5	4.5 ± .5	5.6 ± .5	* 9.0 ± .5	* 9.0 ± .5
7	5.1 ± .5	5.1 ± .5	* 8.0 ± .4	*10.0 ± .8
10	—	5.3 ± .5	—	*14.0 ± 1
14	5.3 ± .5	5.3 ± .5	* 9.1 ± .5	*13.3 ± 1
23	5.4 ± .5	5.1 ± .5	* 9.0 ± .5	*12.0 ± 1

† First day of trypsin treatment.

* Significantly different ($p < .05$) from control mean (day 0).

Underlined mean differs significantly from that of untreated lesion.

Table V shows that the concentration of citrate soluble hydroxyproline in uninjured skin distal to the site of local injury increased some 4-fold in concentration while topical trypsin treatment of the local lesion resulted in a 7-fold increment in this material. After treatment of the lesion for 20 days with trypsin, the dermal concentration of citrate soluble hydroxyproline remained elevated above normal.

Subtraction of the sum of all 3 soluble fractions (0.15 and 0.50M NaCl and citrate) from total hydroxyproline content of the tissue permits calculation of the concentration of the insoluble hydroxyproline (Table V). From these data it may be seen that most of the de-

TABLE V. Citrate soluble and insoluble hydroxyproline concentration (μmoles/g) of rat skin distant from site of croton oil injury with and without topical trypsin treatment.

Days after Injury	Citrate soluble		Insoluble Residue	
	control	trypsin	control	trypsin
0	8 ± .6	8 ± .6	200 ± 20	195 ± 18
2	*25 ± 1.2	*25 ± 1.2	*132 ± 13	*132 ± 13
3†	*30 ± 1.5	*29 ± 1.5	*134 ± 13	*160 ± 16
4	*31 ± 1.6	—	*130 ± 13	—
5	*33 ± 1.7	*59 ± 3.0	*129 ± 13	*156 ± 16
7	*33 ± 1.7	*57 ± 2.6	*128 ± 13	*132 ± 13
10	—	*57 ± 2.6	—	*140 ± 13
14	*34 ± 1.7	*48 ± 2.3	*154 ± 16	*144 ± 14
23	*18 ± .9	*38 ± 2	200 ± 20	*130 ± 11

† First day of trypsin treatment.

* Significantly different ($p < .05$) from control mean (day 0).

__ Underlined mean differs significantly from that of untreated lesion.

crease in hydroxyproline content of uninjured skin distal to the site of injury was accounted for on the basis of a loss in hydoxyproline content of the insoluble residue of the tissue. Topically applied trypsin was without significant effect upon the concentration of this insoluble material.

Despite these changes in dermal hydroxyproline concentration with inflammation there were no significant alterations from normal in either the nitrogen or hexosamine content of the various fractions described above.

Discussion. Collagen contains 13.6% hydroxyproline or about 1 μmole/mg and 18.6% nitrogen or 13.3 μmoles/mg(14). Since this imino acid is characteristic of collagen(15), the mg of collagen per g of shaven dermis may be obtained from the μmoles of hydroxyproline contained in the tissue. Multiplication of these values by 13.3 permits concentration of collagenous nitrogen to be calculated. In view of the essential constancy of the nitrogen content of the lesion with inflammation, the marked decrease in dermal collagen must be compensated for with respect to nitrogen by increases in either non-collagenous protein or various non-protein nitrogen containing materials. Topically applied trypsin was without significant effect upon this local reaction to inflammation.

Associated with the decrease in dermal hydroxyproline, or collagen, with local inflammation was an increase in hexosamine concentration of the lesion. This increase was lessened when the wound was exposed daily to trypsin. By the third day after injury, the untreated lesion contained 65% more hexosamine than the normal skin. Trypsin treated lesions contained only 27% more amino sugar than the control. These increases occurred in the face of an increase in water content of the tissue of less than 12%. In light of the magnitude of the alterations in dermal concentration of collagen and hexosamine with local inflammation, the changes in tissue water content are not sufficient to explain either the increase in the hexosamine or decrease in collagen content of the lesion.

With local trypsin treatment, both the elevation in dermal hexosamine and non-collageneous nitrogen and the decrease in lesion collagen were prolonged beyond those demonstrated by the untreated lesion.

Despite the decreased time required for sloughing of the eschar, the local trypsin treated lesion therefore did not heal chemically as quickly as the untreated control. This finding supports the claim of Castigliano and Rominger(16) that trypsin destroys and digests marginally viable cells in injured tissues, and thereby inhibits healing. It should be emphasized, however, that the trypsin was applied even after the eschar sloughed, when this treatment would not be normally continued. These adverse chemical findings with topical trypsin thereby may not then be equivalent with the routine clinical application of this enzyme.

Menkin(17) indicates that the biological continuity of the skin is destroyed during inflammation by fibrin and other proteins plugging both the lymphatics and the capillaries. These proteins flood the injured tissue as a consequence of the initial increase in capillary permeability(8). As expected, the consequence of this restoration of the biological continuity of the injured tissue would be the appearance of wound products in the circulation. Table II suggests that dermal hydroxyproline, hexosamine and nitrogen drain from the lesion into the sera with topical application of trypsin.

Less than 10% of the nitrogen content of the isotonic saline extract was soluble in 20% trichloracetic acid. Therefore most of the nitrogen found in uninjured skin was not non-protein-nitrogen. Since 20-30% of the dermal collagen was lost from the uninjured skin with local inflammation while total nitrogen content of the tissue was not altered, non-collagenous protein must be replacing the disappearing collagen. This "distant dermal collagen response" to local inflammation must therefore be specific for collagen rather than a general catabolic decrease of all proteins.

If the loss in dermal collagen from skin distant from site of injury was in response to the systemic distribution of either the products of local inflammation or some inflammatory factors released from white blood cells, then the local application of trypsin, which results in wound products entering the circulation, would be expected to increase this response. Since the distant response was actually decreased with topical trypsin, both possibilities would seem unlikely. The insoluble nature of the lipid irritant renders equally untenable the theory that this distant response occurs as reaction to the systemic distribution of either the irritant itself or to some contaminants of the croton oil. Two other major explanations of the etiology of the distant response i.e. 1) a neurally potentiated organ response to local injury and 2) a response to the adrenal hormones provided in reaction to the trauma produced by local injury, occur to the author. It is doubted that topical trypsin would inhibit, even slightly, the nerve transmission of local injury stimuli to the rest of the skin, particularly since the sizes of both the trypsin treated and the control lesion were similar. The trauma produced by the enzyme treated lesion might be minimized by the increased flow of blood elements through the dermal lesion, however. It should be noted that sham adrenalectomy, croton oil and alkali induced injury and small doses of cortisol result in very similar changes in dermal concentration and distribution of hydroxyproline(18).

The extraction of the uninjured skin distal to the site of local injury indicates that inflammation results in a generalized decrease of about 60% in the neutral soluble collagen content of the tissue, and an increase of 400% in the citrate soluble collagen. Trypsin, applied topically to the local lesion, results in the former material decreasing only 45%, while the latter collagen increases 700% above the normal control value. The enzyme treatment did not affect the decrease in concentration of insoluble collagen, however, except that this decrease was prolonged beyond that demonstrated by the enzymatically untreated animals.

Since the increases in dermal content of citrate soluble collagen are never enough quantitatively to account for the loss in insoluble collagen, it is unlikely that this latter type of collagen is being converted into soluble collagen. Further, the circulating concentration of hydroxyproline is not profoundly elevated unless the local lesion is subjected to topical trypsin treatment. Therefore it also appears unlikely that the break-down products of insoluble collagen from the uninjured skin drain into the blood.

Summary and Conclusions. The effects of daily topical application of trypsin upon the dermal chemistry changes produced by croton oil induced local inflammation at the site of injury as well as distant from the site of injury, were compared with enzymatically untreated irritant injected control rats. Locally, trypsin treatment resulted in an inhibition of the increase in lesion hexosamine normally encountered in response to chemically induced inflammation. The collagen content of the lesion decreased independently of topical trypsin application. Although the eschar sloughed 10 days after injury with trypsin treatment, as opposed to the 13 days required by the controls, continued topical trypsin treatment inhibited the chemical healing of the lesion.

The serum nitrogen, hexosamine and hydroxyproline were significantly increased with trypsin treatment; evidence for the increased drainage of wound products into the circulation via proteolytically unblocked capillaries and lymphatics.

Local inflammation resulted in profound and specific decreases in concentration of collagen in uninjured skin distal to site of inflammation. Topical application of trypsin to the lesion appeared to partially inhibit this decrease in dermal collagen distant from the site of local injury. Topical trypsin application increased the amount of citrate soluble collagen found in uninjured skin distant from the site of local injury, but both groups of animals demonstrated a similar and profound loss in insoluble collagen. This dermal collagen loss was prolonged with continued trypsin treatment of the local lesion.

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Salivary Gland Function and Electrolyte Composition in Potassium-Deficient Rats.* (27003)

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Previous investigations(1-6) have shown that dietary deprivation of potassium, in addition to altering electrolyte and water content of specific tissues, may also lead to changes in their function. Alteration in function has been observed in contractile(1-4) and some secretory tissues(5,6). This investigation was undertaken to determine the effects of dietary deprivation of potassium on function and on tissue electrolyte content of another secretory system, the salivary glands, with a view of correlating possible changes in function (altered composition or volume of secretion) with gland electrolyte changes.

Methods. Long-Evans rats, 6 weeks to 4 months of age, weighing 100 to 200 g, were used in these experiments. Experimental animals were placed on a potassium-deficient diet (Nutritional Biochemicals Corp.) composed of cornstarch, 64.2%; casein, 30%; butterfat, 3.5%; calcium carbonate, 1.3%; sodium chloride, 1.0%; and complete vitamin supplements. Potassium content, determined on an ashed sample, was 0.54 mM/100 g of diet. Animals drank tap water (potassium less than .04 mM per liter) or distilled water. Control animals were fed a standard laboratory diet. After 45 days on the diet, animals were fasted 24 hours, lightly anesthetized with Nembutal, and the left parotid, submaxillary, and sublingual glands were removed, individually weighed on a torsion balance, and subsequently dried in Vycor crucibles to determine tissue water content (expressed on a dry weight basis). The duct from one of the remaining salivary glands (in some cases, all 3) was freed from surrounding tissue and cut. Pilocarpine nitrate (2.2 mg) was injected subcutaneously and ensuing secretion was collected, either directly in a small tared vessel(7) or, by micropipette placed at the duct orifice. After saliva collection, the secreting gland was removed for weighing and water and electrolyte analysis. Gland electrolytes were determined on samples dry-ashed (525°C) in Vycor crucibles and are expressed as milliequivalents per kg wet weight (meq/kg

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w.w.) of tissue. Rate of salivary flow is expressed as milligrams of fluid obtained per minute per milligram of gland (mg/min/mg), since flow data included were obtained by weighing saliva collected in a timed interval. Additional values for salivary flow were obtained by measurement of time required to fill a calibrated micropipette, and these volume/time values (per unit mass of gland), and electrolyte concentrations based on them, agree closely with values given. To obviate further the possibility that fluid density differences could affect comparisons of flow and electrolyte composition of the secretions, direct determinations of density were made by weighing known volumes of secretion contained in tared micropipettes. The densities of individual secretions of control and potassium-deficient animals showed differences among themselves, which, for purposes of this investigation, were insignificant: agreement was within 2%, even with weighing errors resulting from necessary use of small volumes. Moreover, density determinations indicate values for specific gravities of the secretions which, for use here, are equivalent to unity. In the experiments, random muscle and blood samples were removed from K-deficient and control animals and electrolyte levels determined so that evidence of potassium deficiency might be established(1). For histological examination, salivary gland samples were preserved in Zenker's-formol and stained with haematoxylin and eosin.

Results. Sodium, potassium and water content of unstimulated parotid, submaxillary and sublingual glands of young rats maintained on a K-deficient diet for 45 days are shown by the data in Table I. These values are compared with those from a group of control animals of similar age maintained on standard laboratory diet. Water content of parotid and submaxillary glands of K-deficient animals was unchanged from that of controls, whereas sublingual glands of K-deficient animals showed a small change in water content (Table I). The potassium level of parotid and submaxillary glands in K-deficiency was lower and sodium higher than in controls; in sublingual, however, sodium and potassium did not change from control levels, even when the small difference in water content between control and experimental glands was taken into account.

TABLE I. Effect of Potassium Deficiency on Sodium and Potassium Content of Unstimulated Rat Salivary Glands.

	K-Deficient Mean ± S.E.*	Control Mean ± S.E.	Signif. of Diff.
Parotid	32 Rats	14 Rats	P
Na	53.7 ± .97	45.5 ± 1.74	.0000
K	64.4 ± .97	68.0 ± 1.33	.0000
Water/Dry	2.5 ± .09	2.5 ± .07	—
Submaxillary	31 Rats	14 Rats	—
Na	49.0 ± 1.73	36.4 ± 1.54	.0000
K	79.0 ± 1.36	89.0 ± 2.02	.0000
Water/Dry	3.2 ± .08	3.3 ± .07	—
Sublingual	24 Rats	10 Rats	—
Na	41.8 ± 2.05	41.2 ± 2.10	.3855
K	69.5 ± .94	73.4 ± 2.34	.1151
Water/Dry	3.0 ± .14	3.5 ± .036	.0012

* Mean and standard error, expressed for Na and K as meq/kg w.w.

Rate and duration of salivary flow from submaxillary and parotid glands of K-deficient animals were markedly lower than from glands of control animals, following maximal stimulation with pilocarpine. Individual submaxillary flow rates (13 K-deficient and 10 control animals), calculated from volumes of fluid obtained in the first 3 to 7 minutes of flow following pilocarpine administration, are shown in Fig. 1. Flow rate of submaxillary saliva averaged .072 ± .013(S.E.) mg/min/mg in the control group and .027 ± .006 mg/min/mg in the K-deficient group ($P = .0013$). Not included are results of 8 experiments in which no measurable flow from submaxillary gland of K-deficient animals could be obtained. Representative individual flow rates from parotid gland of control and potassium-deficient animals are also shown in Fig. 1. From these data, and additional values, an average parotid flow rate of .058 ± .006 mg/min/mg was obtained for control (17 animals) and .018 ± .002 mg/min/mg for K-deficient (14 of 30 animals from which flow was obtainable) animals ($P < 10^{-6}$). The reduced flow rate, found to be characteristic in K-deficiency, is probably not due to an increased dose threshold, since increased pilocarpine dosage, adminstered to experimentals and controls, did not modify the effect. Electrolyte levels of parotid and submaxillary secretions from the K-deficient animals were generally similar to those of controls, although an occasional ele-

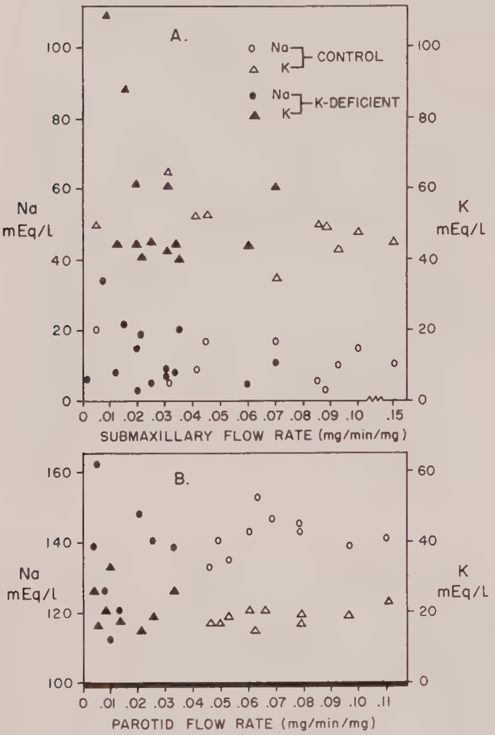

FIG. 1. Flow rate and electrolyte levels of parotid and submaxillary secretions from control and K-deficient animals during first 3 to 7 min. interval of secretion following pilocarpine stimulation.

vated potassium did occur in both secretions from the deficient animals (Fig. 1). This tendency toward elevation of potassium was accentuated when the salivas were collected only for the first one to 2 minutes after start of flow. Sublingual saliva sodium and potassium levels in the 2 cases where adequate saliva volume was recovered, were similar to those of controls(7).

Histological specimens prepared from a few potassium-deficient glands showed no striking changes although a greater number of cytoplasmic vacuoles were evident.

Discussion. It is clear from these investigations that, although potassium-deficiency effected an altered sodium-potassium balance of the parotid and submaxillary glands of rat, this modification did not approach, even in the submaxillary, the marked electrolyte changes induced in muscle(1). Nonetheless, associated with this glandular potassium-deficiency is one striking functional change: capacity to secrete fluid is much reduced. Histological examination did not reveal changes that could account for this diminished secretory capacity. A similar functional change has been reported for kidney(5). In another secretory organ, stomach, secretory volume is reported to be unchanged in potassium deprivation, but sodium and potassium levels of this secretion are greatly modified(6). Mechanisms mediating changes observed in potassium-deficiency are not well understood. Some similarity between effects of potassium deficiency and administration of adrenocortical hormones has been observed(8,9).

In view of numerous investigations(7,10) which suggest a dependence of secretory fluid electrolyte levels on gland levels, it may be noted that gland level changes in potassium deficiency give no evidence of being reflected in corresponding changes in secretion electrolyte levels.

Summary. Modification of sodium and potassium levels of submaxillary and parotid, but not sublingual, glands was achieved by reduction of dietary potassium. Submaxillary gland exhibited a decrease in potassium of approximately 10 meq/kg w.w. when compared with normal animals of the same age, and parotid approximately 4 meq/kg w.w. Sodium increased in both glands (8 meq/kg w.w. for parotid; 13 for submaxillary). The major functional change observed in potassium deficiency was a marked reduction in fluid flow rate; electrolyte levels of the secretions were not influenced by changed gland levels.

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Effect of Trypsin Inhibitors on Shwartzman Phenomenon.* (27004)

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Despite the similarity of some of the pathologic findings in varied abnormal states such as the Shwartzman phenomenon, the Arthus phenomenon and other antigen-antibody reactions, and certain types of allergy and shock, neither a common factor nor a fundamental mechanism responsible for the pathogenesis of these reactions has been established. Some of these reactions may be induced by bacterial endotoxins which, in all likelihood, act indirectly via one or more intermediate steps. There is increasing evidence that the mechanism of these phenomena involves a complex chain of reactions in which enzyme activities may play important roles. Intracellular proteolytic enzymes have been considered essential factors in production of the Shwartzman phenomenon(1,2) and trypsin was reported to potentiate this phenomenon(3). In addition the blood coagulation process, an important element in the Shwartzman reaction(4,5), is also influenced by proteolytic enzymes. Besides, bacterial endotoxins induce fibrinolytic activity *in vivo*(6,7,8), and are also thought to activate other proteolytic enzymes which are distinct from the plasminogen-plasmin system (9). Participation of such enzymes have been suggested in the mechanism of endotoxin induced shock(9). Recently, *in vitro* potentiation of serum proteolytic activity by endotoxins was also reported(10). These proteolytic enzymes might activate plasma kininogens which in turn effect tonicity and permeability of blood vessels. In view of these considerations a study of the effect of trypsin inhibitors on the Shwartzman phenomenon was undertaken (11).

Materials: 160 albino female rabbits, weighing between 1.7 and 2 kg, were used in these experiments. They received Rockland rabbit ration, (antibiotic-free) and water *ad libitum* and were housed in air-conditioned animal rooms at about 65°F. The abdominal skin was depilated with an electric clipper.

The inhibitors employed were crystalline pancreatic trypsin inhibitor, crystalline soybean trypsin inhibitor, and crude soybean trypsin inhibitor obtained from Mann Research Laboratories.

Solutions of *E. coli* endotoxin (Difco) in concentrations from 3 μg/ml to 500 μg/ml were used for skin preparation and 250 μg/ml for all provocative intravenous injections. A volume of 1 ml was used for each intradermal or intravenous injection. All solutions in these experiments were made in pyrogen-free normal saline.

Method: Exp. I. In 34 rabbits varying amounts of endotoxin ranging from 6 to 500 μg were injected intradermally at different sites along one flank of each animal. The same amounts of endotoxin mixed with one of the trypsin inhibitors were injected at corresponding sites of the other flank. The amount of trypsin inhibitor in each injection varied from 6 μg to 10 mg. Crystalline pancreatic trypsin inhibitor was used for 6 rabbits (20 sites), crystalline soybean trypsin inhibitor for 3 rabbits (9 sites) and crude soybean trypsin inhibitor for the remaining animals. The provocative endotoxin injection was intravenously administered 24 hours later.

Exp. II. 6 rabbits in a control group and 10 rabbits in a second group were injected intradermally at various sites of the abdominal skin with 6, 12, 25 and 50 μg of endotoxin. Within a period of 5 minutes before or after these intradermal injections the second group received 500 μg of a trypsin inhibitor in 1 ml of saline intravenously. Crude soybean trypsin inhibitor was used for 2 rabbits and crystalline soybean trypsin inhibitor for the remaining 8 animals. The provocative intravenous injection of endotoxin was administered 24 hours later to both groups.

Exp. III. Various amounts of endotoxin ranging from 12 to 100 μg were intradermally injected at various sites along one flank of 47 rabbits. The same amounts were injected at

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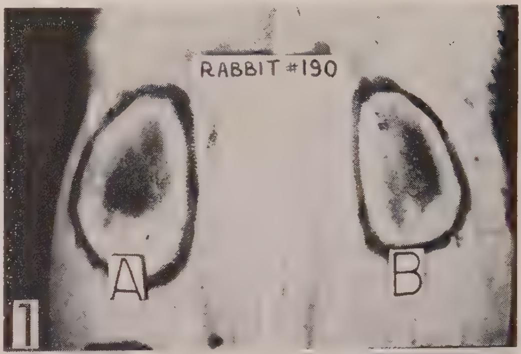
* Aided by Grants from U.S.P.H.S., from The Joseph and Helen Yeamans Levy Foundation, and Saul Singer Foundation.

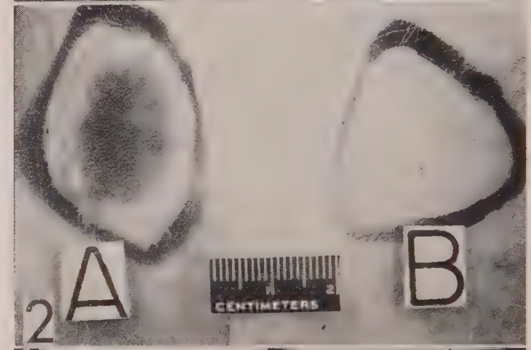
corresponding sites of the other flank. Each injection site was carefully marked with a circle. Twenty-four hours later 0.5 ml of saline was injected in all sites of one flank which served as controls. The same volume of saline containing 50 μg to 3 mg of one of the trypsin inhibitors was injected in the corresponding sites of the other flank. Crystalline soybean trypsin inhibitor was injected in 3 rabbits (7 sites), crystalline pancreatic trypsin inhibitor in 2 rabbits (2 sites) and crude soybean trypsin inhibitor in the remaining animals. The provocative injection of endotoxin was administered within a period of 30 minutes before or after the intradermal inhibitor injection.

Exp. IV. Twenty-eight rabbits in the control group and 37 in a second group were injected intradermally at different sites of the abdominal skin with various amounts of endotoxin ranging from 3 to 100 μg. The provocative intravenous injection of endotoxin was administered 24 hours later to both groups. The second group received in addition 500 μg of one of the trypsin inhibitors in 1 ml of saline intravenously simultaneously with or at intervals ranging from 5 to 120 minutes before or after the provocative endotoxin injection. Of the 37 rabbits in this group 4 were treated with crystalline soybean trypsin inhibitor, 3 with crystalline pancreatic trypsin inhibitor and 30 with crude soybean trypsin inhibitor.

Results: The skin reactions in all experiments were read 5 hours after the provocative endotoxin injection.

Exp. I. Trypsin inhibitors administered intradermally mixed with the endotoxin skin preparatory dose. In the majority of the animals (74%) the intradermal injection of trypsin inhibitors in combination with endotoxin did not influence the skin preparatory ability of the latter. The number and intensity of positive reactions for each preparatory dose of endotoxin was about the same in both control and inhibitor injected sites (Fig. 1). (In 19% of the animals the reaction in the sites injected with trypsin inhibitors was slightly weaker and in 7% slightly stronger than in the corresponding control sites. These variations in the intensity of reaction are statistically insignificant.) No differences were noted in respect to type or amount of trypsin inhibitor employed. Incubation of the endotoxin-trypsin inhibitor





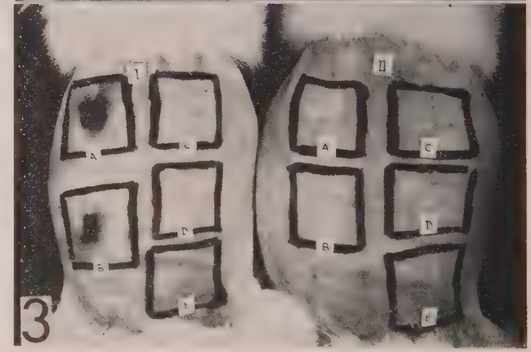


FIG. 1. Site A intradermally injected with 50 μg endotoxin alone. Site B intradermally injected with 50 μg endotoxin mixed with 50 μg trypsin inhibitor. Equal Shwartzman reactions after provocative endotoxin dose.

FIG. 2. Sites A and B intradermally injected with 50 μg endotoxin. 24 hours later (30 minutes prior to provocative endotoxin injection) site A intradermally injected with saline (1 ml) and site B with trypsin inhibitor (2 mg in 1 ml saline). Reaction in site B inhibited.

FIG. 3. Sites A, B, C, D and E in both rabbits intradermally injected with 50, 25, 12, 6 and 3 μg endotoxin respectively. Control rabbit I (left) after the provocative endotoxin dose produced positive reactions at sites A and B. In rabbit II (right) which received 500 μg trypsin inhibitor intravenously 15 min. prior to the provocative endotoxin injection, reactions were inhibited in all sites.

TABLE I. Effect of trypsin inhibitors on the local Shwartzman phenomenon.

Endotoxin intradermal dose (μg)	% positive skin reactions		% strong positive skin reactions*	
	Control rabbits	Trypsin inhibitor treated rabbits	Control rabbits	Trypsin inhibitor treated rabbits
100	100 (5/5)**	100 (8/8)	60.0 (3/5)***	50.0 (4/8)
50	96.6 (28/29)	75.0 (27/36)	36.6 (17/29)	8.3 (3/36)
25	89.3 (25/28)	56.7 (21/37)	50.0 (14/28)	10.8 (4/37)
12	75.0 (21/28)	29.7 (11/37)	14.3 (4/28)	0 (0/37)
6	47.8 (11/23)	17.2 (5/29)	0 (0/23)	0 (0/29)
3	30.4 (7/23)	3.7 (1/27)	0 (0/23)	0 (0/27)

* 3+ or 4+ reaction. (Positive reactions were graded from 1+ to 4+ according to intensity and size of the hemorrhagic area.)

** Number of positive reactions/number of animals.

*** Number of strong positive reactions/number of animals.

mixture for 30 minutes at 37°C before administration to 6 of the animals of this experiment did not alter the results.

Exp. II. Trypsin inhibitors administered intravenously at about the time of skin preparation. Trypsin inhibitors injected intravenously at intervals ranging from 1 to 5 minutes before or after skin preparation did not inhibit production of the local Shwartzman phenomenon. In a few cases there were differences in the intensity of the reaction between control and trypsin inhibitor treated groups, but these differences did not exceed normal variations in reactivity of individual animals. No differences were noted in respect to the inhibitor employed or to the time relationship between skin preparation and inhibitor administration.

Exp. III. Trypsin inhibitors injected intradermally 24 hours after skin preparation into the primed skin sites. In many animals trypsin inhibitors abolished the reaction completely (in 39 of 99 injection sites) (Fig. 2), or limited the skin hemorrhage to a zone surrounding the site of inhibitor injection. In the corresponding control sites which were injected with normal saline, the reaction was never completely inhibited. In some cases, however, there was a small area around the puncture mark of the saline injection which remained pale.

In this experiment although trypsin inhibitors influenced the reaction in a considerable number of animals, there was no apparent correlation between degree of inhibition and amount of inhibitor used or dose of preparatory endotoxin injection. The time relationship between inhibitor injection and administration of the provocative endotoxin dose did not influence the results.

Exp. IV. Trypsin inhibitors administered intravenously at about the time of provocative endotoxin injection. In the group treated with trypsin inhibitors the reactions were considerably inhibited (Fig. 3). Table I summarizes results in this experiment. All concentrations of endotoxin except the 100 μg one, produced fewer positive reactions in the inhibitor treated group than in the control animals. As concentration of endotoxin decreases the effect of the trypsin inhibitor is more striking. In the inhibitor treated group, not only was the number of positive reactions considerably smaller than in the control group, but also the intensity of positive reactions was less pronounced (Table I). Statistical evaluation of the results obtained in this experiment indicates that the differences in reactions between controls and inhibitor treated animals are significant at high levels of confidence. ($P = 0.001$-0.01 for differences in frequency of reaction; $P = 0.03$-0.04 for differences in intensity of reaction). The effect of trypsin inhibitors was most pronounced when administered before the provocative endotoxin injection, less pronounced when administered simultaneously and least evident when administered after the provocative endotoxin injection. No differences were noted in respect to the inhibitor employed.

In addition to the inhibition of the local Shwartzman reaction trypsin inhibitors also prevented renal changes seen in the generalized Shwartzman phenomenon. These changes which were present in 10 out of 28 control rabbits were found in only 1 of 37 inhibitor treated rabbits.

Discussion: The experiments herein reported indicate that trypsin inhibitors administered intravenously at about the time of the provocative endotoxin injection inhibit the local Shwartzman phenomenon. It is not unreasonable to assume that trypsin inhibitors influence the Shwartzman phenomenon by interfering with proteolytic enzymes which apparently play a role in the mechanism of this phenomenon. Injection of trypsin inhibitors directly into the primed skin sites 24 hours after skin preparation also influenced the local Shwartzman reaction. To a limited extent this effect might be attributed to physical factors related to the volume of the injected fluid since saline injections directly into control sites also, in a few instances, inhibited hemorrhage in a focus about the injection point.

The inhibitors mixed with the intradermal endotoxin preparatory dose, or given intravenously at time of skin preparation, failed to inhibit the Shwartzman phenomenon. This lack of effect at the time of skin preparation may be due to the fact that one or several enzymes which are inhibitable by trypsin inhibitors are not present at this time, being activated or released only after a lapse of time following skin preparation or after the provocative endotoxin injection. A similar delay in activation has also been observed with the permeability factor in diluted plasma(12). Other possibilities to be considered are that proteolytic enzymes are present at the time of skin preparation but their inhibition at this phase of the phenomenon is not critical for development of the reaction or an excess of autochthonous inhibitor is normally present at time of skin preparation and injection of additional inhibitor has no effect.

We observed visceral lesions, after eliciting the local Shwartzman reaction which were indistinguishable from those seen in the generalized Shwartzman phenomenon; this in all likelihood was due to escape of endotoxin from the local skin sites into the lymphatics and in turn into the blood stream. Trypsin inhibitors intravenously administered at time of the provocative endotoxin injection prevented development of these lesions. It may be postulated that proteolytic enzymes are involved in both the local and generalized Shwartzman reaction. The fact that trypsin inhibitors influenced the Shwartzman reaction while epsilon aminocaproic acid (EACA), which primarily prevents conversion of zymogens into the active enzymes failed to do so(13), favors the hypothesis that enzymes involved in the Shwartzman phenomenon are released in their active form from cells or from enzyme-inhibitor complexes. This does not completely exclude zymogen activation which is not affected by EACA. The enzymes involved in the Shwartzman phenomenon may produce tissue changes directly or through release of histamine, heparin, serotonin and/or activation of plasma kinins(14, 15). These factors which have vasomotor effects and can alter the blood coagulation process and vascular permeability may be essential for this phenomenon and other tissue reactions mentioned previously.

Summary. 1 Trypsin inhibitors injected intravenously at about the time of the provocative endotoxin injection, partially or completely inhibited the local Shwartzman phenomenon and prevented development of renal lesions (as seen in the generalized Shwartzman phenomenon). 2 Inhibitors administered intradermally mixed with the endotoxin skin preparatory dose, or intravenously at time of skin preparation, did not influence the reaction. 3 The data presented suggest that activated proteolytic enzymes are involved in the Shwartzman phenomenon. 4 The role of these enzymes in the pathogenesis of the phenomenon is discussed. Alterations of the clotting mechanism and vascular changes brought about by release of serotonin, histamine and activation of plasma kinins have been considered.

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Hyaluronidase Inhibitor and Trypsin Inhibitor in Preparations from Human Urine.* (27005)

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Shulman(1) and Astrup et al.(2) have elaborated methods for isolating the trypsin inhibitor in human urine, and preparations with high inhibitor content have been obtained. These preparations also inhibit chymotrypsin and plasmin.

Recently it was observed that the hyaluronidase in human urine is strongly inhibited at pH 4.63 by a substance in the urine(3). The present communication shows that hyaluronidase is also inhibited by preparations purified by Astrup et al., and that concentrations of trypsin inhibitor and hyaluronidase inhibitor in preparations of different degrees of purification, parallel each other.

Methods. Trypsin inhibitor concentrations were estimated on casein by the biuret method (4). One trypsin inhibitor unit is the amount which inhibits 1 μg crystalline trypsin, containing 24.4 Anson units per gram (Novo Laboratories, Copenhagen).

Five preparations containing varying concentrations of trypsin inhibitor were prepared from human pregnancy urine and placed at our disposal by Dr. Tage Astrup. Prep. I contained 56.4, Prep. II 187.5, Prep. III 234, Prep. IV 333 and Prep. V 612 trypsin inhibitor units per mg. Preparations I to IV were prepared as before(2). Preparation V had been further purified (Astrup, to be published).

Hyaluronidase activity was estimated viscosimetrically(3), using USP Reference Standard hyaluronidase (bovine testis). Thirty μg of hyaluronidase preparation and different amounts of Prep. V (Fig. 1) or of preparation I to IV (Table 1), dissolved in 30 μl acetate buffer (pH 4.63) containing albumin, were added to 150 μl of the substrate (details in (3)).

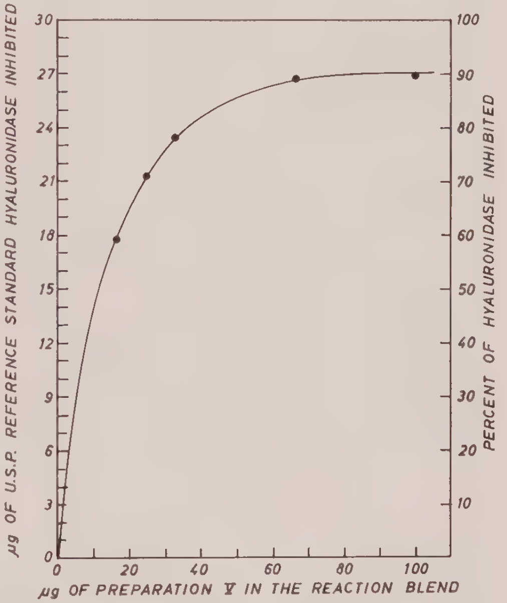

FIG. 1. Inhibition of USP Reference Standard Hyaluronidase by different amounts of a purified urinary trypsin inhibitor preparation containing 612 trypsin inhibitor units per mg (Preparation V). *Abscissa*, μg of trypsin inhibitor preparation in the reaction blend. *Ordinate*, Inhibited amount of hyaluronidase expressed in % of total amount present and μg of hyaluronidase inhibited.

Results. Fig. 1 shows that preparation V, the purest of the trypsin inhibitor preparations, inhibits testis hyaluronidase. The curve indicates a dissociable binding between hyaluronidase and hyaluronidase inhibitor. It is inter-

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* Supported by grants from Danish State Research Foundation.

TABLE I. Contents of trypsin inhibitor and hyaluronidase inhibitor in preparation I to IV.

Trypsin inhibitor preparation	Amt. of preparation in reaction blend, mg	Units of trypsin inhibitor in reaction blend a	μg of hyaluronidase inhibited b	Amt of hyaluronidase inhibitor* in reaction blend c	c/a
I	.100	5.6	11.4	8	1.4
IV	.030	10.00	18.5	18	1.8
II	.100	18.8	23.3	32	1.7
III	.100	23.4	24.9	42	1.8

* expressed as μg of preparation V as estimated by interpolation on the curve in Fig. 1. (c/a for preparation V is 1.6).

esting that hylauronidase present in human urine also is influenced by an inhibitor in a dissociable manner(3).

Table 1 compares concentrations of trypsin inhibitor in Preparations I to IV with estimated amounts of hyaluronidase inhibitor. Column *a* shows amounts of trypsin inhibitor present in the reaction mixtures calculated from the activities of the trypsin inhibitor preparations. Column *b* shows amounts of hyaluronidase inhibited as estimated in the experiments. Column *c* shows amounts of hyaluronidase inhibitor present as calculated from the values in column *b* by interpolation on the curve in Fig. 1 and expressed as μg of preparation V. The last column shows the proportion between amounts of hyaluronidase inhibitor found and trypsin inhibitor present in the reaction mixtures. It is seen that approximately identical proportions are obtained.

It seems permissible to conclude that concentrations of trypsin inhibitor and hyaluronidase inhibitor parallel each other in the different preparations. Apparently the hyaluronidase inhibitor and trypsin inhibitor behave identically during the process of purification, suggesting that the two effects are caused by a single compound. This possibility is interesting in view of the relationship between urinary trypsin inhibitor excretion and pituitary-adrenal hormones as demonstrated previously (4,5).

Summary. Trypsin inhibitor preparations isolated from human pregnancy urine inhibit hyaluronidase. The activity curve indicates formation of a dissociable compound. The trypsin inhibitor concentration in preparations of different purity parallels the content of hylauronidase inhibitor suggesting identity of the 2 inhibitors.

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Acute Effects of 9α-fluorohydrocortisone and 2-methyl-9α-fluorohydrocortisone on Renal Clearances of Sodium and Potassium in Man.* (27006)

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Before beginning a study of the modifying effects of sodium-retaining steroids upon mercurial diuresis, it was necessary to establish the usual pattern of response to the steroids in normal human subjects. The steroids used were 9 α-fluorohydrocortisone (FF) and 2-methyl-9 α-fluorohydrocortisone (2MeFF). The latter compound has been shown by Liddle and his associates(1) to exert a more potent sodium-retaining effect than aldosterone. The acute

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effects of these compounds upon renal hemodynamics and the renal clearances of sodium, potassium, and chloride in salt-loaded and salt-depleted humans are reported here.

Method. Standard acute renal clearance techniques were employed with 14 human male subjects without evidence of cardiovascular or renal disease. Seven of the subjects were salt-loaded for a period of 3 or more days prior to study by addition of 10 g of sodium chloride daily to the standard hospital diet. Seven subjects were salt-depleted by administration of a mercurial diuretic followed by 3 or more days of a 500 mg sodium diet prior to study. In all subjects, inulin and p-aminohippurate were infused intravenously in a 0.9% sodium chloride solution at a rate of 5 ml/min by a Sigma constant infusion pump. The subjects were well hydrated and in the morning post-absorptive state at time of study.

After 30 minutes of infusion, three 20 minute control clearance periods were obtained utilizing an indwelling bladder catheter and distilled water and air washouts. Following these control periods, the steroids were given intravenously to 12 of the subjects. 2MeFF was given to 6 subjects, 3 salt-loaded and 3 salt-depleted, as a single dose of 0.5 to 0.9 mg (8.1 to 10.6 μg/kg). FF was given to 6 subjects, 3 salt-loaded and 3 salt-depleted, in a priming dose of 5 mg, and in 4 subjects this was followed by a sustaining dose of 3 mg/hr. Total dosage of the latter compound varied from 5.0 to 19.5 mg (67 to 238 μg/kg). Two of the 14 subjects served as controls, 1 salt-depleted and 1 salt-loaded as described. The technique with these 2 subjects was identical with that utilized with the other 12, except that no steroid was given.

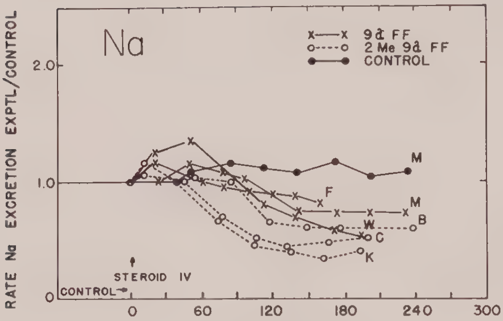
Following administration of the steroid, eight 30 minute urine collections were obtained. Blood was drawn at appropriate intervals for standard clearance calculations. Renal clearances of inulin, p-aminohippurate, sodium, potassium, and chloride were determined. The analytical methods employed were those of Schreiner(2) for inulin, Smith et al(3) for p-aminohippurate, Schales and Schales(4) for chloride, and flame photometry for sodium and potassium with the Baird internal standard flame photometer.

Results. 1. Renal hemodynamics. The data in all 14 experiments are summarized in Table I. Neither steroid significantly influenced the glomerular filtration rate (inulin clearance), with the exception of subject A.C., No. 11, who was salt-depleted and received 2MeFF. His C_{In} rose from 106 to 123 ml/min, an increase of 16% in glomerular filtration.

A slight but consistent depression of C_{PAH} was noted in all subjects during experimental periods, except subject A.C., in whom there was a rise of C_{PAH} from 526 to 726 ml/min, an increase of 38% in effective renal plasma flow. The magnitude of the C_{PAH} depressions (averaging 9%) and the fact that similar C_{PAH} depressions were noted in the 2 control subjects suggest that factors other than steroid administration were responsible for these small changes in effective renal plasma flow.

2. Sodium excretion. With the exception of subject J.F., No. 1, a significant decrease in rate of sodium excretion was produced in all subjects with both compounds. Within the 4 hour experimental period, minimum rate of excretion of sodium ranged from 0.76 to 0.20 of the rate excreted during the control period. The decrease in rate of sodium excretion was relatively less with FF during salt-loading as compared to the salt-depleted state. With 2MeFF, comparable reduction in sodium excretion occurred during both the salt-depleted and salt-loaded states. In the 3 salt-loaded subjects receiving FF, sodium excretion declined to 0.53, 0.76 and 0.95 of the rate excreted during the respective control periods, whereas in similarly loaded subjects receiving 2MeFF, sodium excretion was reduced to 0.39, 0.42, and 0.58 of the rate during control periods. In salt-depleted subjects receiving FF, sodium excretion dropped to 0.14, 0.25, and 0.57 of the rate excreted during control periods. Similar values with 2MeFF were 0.37, 0.40 and 0.44. By contrast, in both the salt-loaded and salt-depleted control subjects, sodium excretion increased during the experimental periods, reflecting the additive effect of administration of 0.9% NaCl intravenously throughout the experiment.

Fig. 1 gives the timed data in all 7 salt-loaded subjects indicating the relative effects of both compounds upon sodium excretion. The data on the ordinate are plotted as follows:



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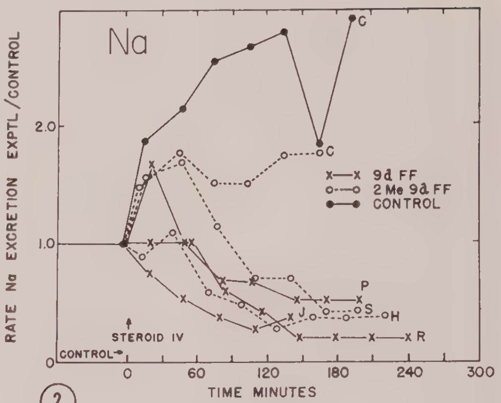

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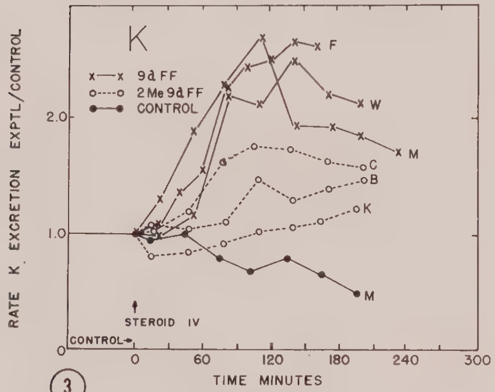

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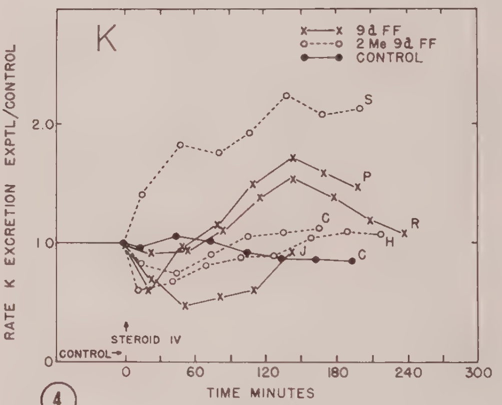

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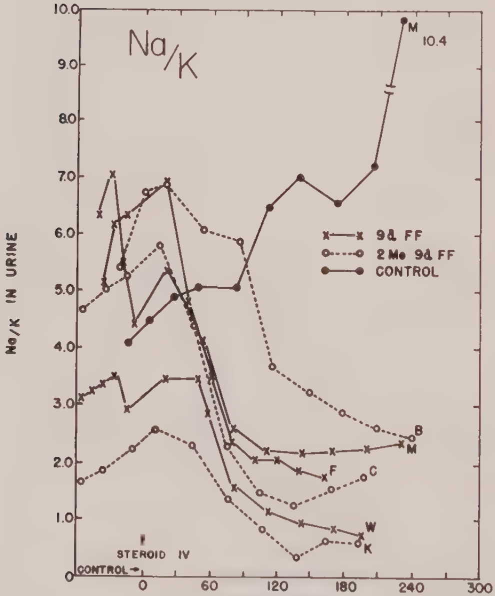

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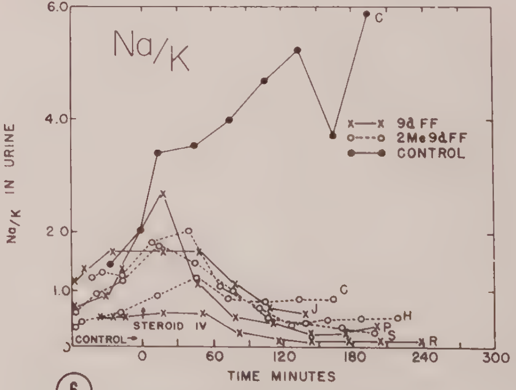

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$$\text{Exp.}\ \frac{\text{Sodium excreted}}{\text{Sodium filtered}}\ \text{Cont.}\ \frac{\text{Sodium excreted}}{\text{Sodium filtered}},$$

which corrects for minor fluctuations of GFR and plasma concentrations. The control sodium excretion in these subjects ranged between 250 and 500 μeq/min, being fairly constant for each subject. Significant depression of sodium excretion began between one and 2 hours after administration of steroids. Rate of excretion of sodium reached the lowest point between 2 and 3 hours after administration, and effectiveness was still evident at the end of the 4 hour experimental period with both compounds. 2MeFF, given in a lower dosage than FF, produced an earlier and greater effect upon sodium excretion.

Fig. 2 presents similar studies in the 7 salt-depleted subjects. The control sodium excretion in these subjects ranged from 45 to 125 μeq/min, being fairly constant for each subject. Of interest is the increase in sodium excretion during the first hour in 2 of the subjects on 2MeFF and 1 on FF. Only in subject A.C., No. 11, with the sustained increase in sodium excretion, was there a significant rise in GFR and renal plasma flow. All patients receiving steroid excreted sodium at a rate much lower than the control patient.

3. Potassium excretion. The greatest increase in rate of potassium excretion occurred in salt-loaded subjects receiving FF, with peak values ranging from 2.12 to 3.08 times the rate of excretion during the control period (Table I). Salt depletion reduced this effect considerably, with actual potassium retention in one subject, H.J., No. 4. 2MeFF produced considerably less kaliuresis in both the salt-loaded and salt-depleted subjects with peak values ranging from 1.01 to 1.64 times control values.

Fig. 3 demonstrates the time relationship of the kaliuretic effects of both compounds in salt-loaded subjects. FF produced an earlier kaliuresis with considerably greater peak effects. The onset was almost immediate with this compound, and peak effects were attained in about 2 hours. With 2MeFF, the kaliuresis started in about one hour and increased gradually to much lower peak values. In contrast, the control subject who received no steroid, N.M., No. 13, revealed a progressive decline in rate of excretion of potassium as compared to the control period.

The modifying effects of salt depletion are seen in Fig. 4. The kaliuretic effects were variable with both compounds. Only one subject with 2MeFF showed an early sustained kaliuresis. Five other subjects revealed an early decrease in rate of potassium excretion, and, of these, only 2 showed later mild kaliuretic effects.

4. Chloride excretion. The effects upon chloride excretion were variable; in general, these reflected the summation of the effects of the steroids upon sodium retention and potassium excretion.

5. Urine Na/K ratios. The ratios of Na/K excretion in the urine are presented in Fig. 5 and 6. The most marked decreases in urinary Na/K are seen in the salt-loaded subjects. With FF, the lower ratios primarily represent marked kaliuresis, whereas similar ratios with 2MeFF reflect primarily sodium retention. By contrast, in both control subjects the urinary Na/K rose during experimental periods.

6. Plasma electrolyte concentrations. During the 4 hours after administration of the steroids, there were insignificant changes in plasma concentrations of sodium, chloride and potassium.

Discussion. It is apparent from these studies that, with the exception of one salt-depleted subject, intravenous administration of FF and 2MeFF in the dose range utilized exerted no discernible effects upon glomerular filtration rate. The changes in sodium and potassium excretion induced by these steroids appear to be primary tubular effects. A dissociation be-

FIG. 1. Rate of sodium excretion in salt-loaded patients; ratio of experimental period to control period.

FIG. 2. Rate of sodium excretion in salt-depleted patients; ratio of experimental period to control period.

FIG. 3. Rate of potassium excretion in salt-loaded patients; ratio of experimental period to control period.

FIG. 4. Rate of potassium excretion in salt-depleted patients; ratio of experimental period to control period.

FIG. 5. Ratio of sodium to potassium excretion in salt-loaded patients.

FIG. 6. Ratio of sodium to potassium excretion in salt-depleted patients.

TABLE I. Effects of 9α-fluorohydrocortisone and 2-methyl-9α-fluorohydrocortisone on renal hemodynamics and electrolyte excretion in salt-loaded and salt-depleted subjects.

									Peak Effects Exp./Control						Urine Na/K		
			Subject	Total Dose (mg.)	C_{In} Control	ml/min (mean) Exp.	C_{PAH} Control	ml/min (mean) Exp.	Sodium Filtered	Sodium UV_{Na}	Potassium Filtered	Potassium UV_K	Chloride Filtered	Chloride UV_{Cl}	Control (mean)	Exp. (Peak)	Exp./-Control
FF	Salt Loaded	1	J.F.	5.0	101	107	877	721	1.16	.95	1.13	3.08	1.08	1.21	5.86	1.76	.30
		2	J.M.	5.0	115	117	593	551	1.06	.76	.99	3.00	1.07	.98	5.80	2.17	.37
		3	W.W.	16.7	138	138	739	615	1.11	.53	.86	2.12	1.13	1.04	3.25	.74	.23
	Salt Depleted	4	H.J.	12.5	78	77	448	420	.85	.25	.90	.83	.92	.40	1.46	.57	.39
		5	J.R.	19.5	108	113	676	648	1.13	.14	1.02	1.55	1.08	2.18	.53	.06	.11
		6	C.P.	11.1	118	131	777	706	1.14	.57	1.14	1.87	1.13	2.21	.99	.26	.26
2MeFF	Salt Loaded	7	R.C.	.5	134	134	1096	963	.95	.42	.90	1.59	1.01	.63	5.00	1.29	.26
		8	N.K.	.9	108	108	664	609	1.06	.39	.97	1.18	1.04	.71	1.89	.37	.20
		9	P.B.	.7	133	135	593	559	1.00	.58	.86	1.28	1.06	.67	6.36	2.44	.38
	Salt Depleted	10	U.H.	.7	89	94	497	484	1.02	.40	1.00	1.01	1.15	1.86	.95	.49	.52
		11	A.C.	.7	106	123	526	726	1.18	.37	1.12	1.23	1.28	2.67	.45	.85	1.89
		12	H.S.	.7	94	91	480	442	1.01	.44	1.03	1.64	1.08	1.52	1.27	.31	.24
Control	Salt Loaded	13	N.M.	—	124	119	545	492	.92	1.12	.95	.46	.92	1.13	4.47	10.37	2.32
	Salt Depleted	14	J.C.	—	98	114	318	306	1.20	2.88	1.26	1.16	1.23	4.00	1.75	5.92	3.38

In the C_{In} and C_{PAH} columns, mean value of three 20 min clearance periods is given in control column, and mean of six to eight 30 min clearance periods is expressed in experimental column. In the electrolyte columns, the experimental values and mean control values are used to calculate the ratios Exp./Control. C_{In} and C_{PAH} data are corrected to ideal surface area of 1.73m^2 with the exception of subject C.P.

tween the effects upon potassium and sodium excretion is present, 2MeFF having a relatively greater sodium-retaining effect and FF a greater potassium-losing effect. The urinary Na/K ratios may, therefore, be misleading when used as a sole guide of effectiveness. The kaliuretic effects of the steroids used appear to be blunted by prior salt depletion and augmented by salt loading. This might be explained by the variation of endogenous aldosterone excretion caused by salt loading and depletion.

In one subject in whom a paradoxical, sustained increase in sodium excretion occurred following intravenous 2MeFF, significant elevations in GFR and renal plasma flow were present. In this subject, the natriuresis associated with increased filtered loads of sodium predominated over the sodium-retaining effects of this potent electrocorticoid.

Following intravenous administration, the electrolyte effects of both steroids became manifest within one and 2 hours and were still present at the end of 4 hours.

Conclusion. 1. Intravenous administration of 9α-fluorohydrocortisone and 2-methyl-9α-fluorohydrocortisone in doses sufficient to produce profound electrolyte effects in 6 salt-depleted and 6 salt-loaded subjects produced no significant change in glomerular filtration rate, with the exception of one salt-depleted subject, in whom there was an increase in both glomerular filtration rate and effective renal plasma flow following 2MeFF. 2. 2MeFF had a more profound sodium-retaining effect than did FF. Even in salt-depleted subjects, the sodium-retaining effects of both compounds were demonstrable. 3. FF had a more profound potassium-losing effect than did 2 MeFF. Kaliuresis was greater in the salt-loaded than in the salt-depleted subjects. 4. Plasma concentrations of sodium, potassium, and chloride did not change significantly during the 4 hour study after intravenous administration of the steroids.

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Possible Relations of Hypoglycaemia Induced by Mono-amine Oxidase Inhibition and Angina Pectoris.† (27007)

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The initially favorable results of the value of the mono-amine oxidase inhibitors in treatment of angina pectoris have latterly been tempered by further experiences of their use(1), and the response to therapy is probably, at best, unpredictable. However, it is of interest that Weiss et al.(2), using one of these drugs, iproniazid, in treatment of depressive patients, showed that those patients whose appetite, weight and sense of well being improved, demonstrated a definite hypoglycaemic response to the drug. It occurred to us that a similar correlation with hypoglycaemia may also exist in patients who showed improvement in the symptoms of angina pectoris as a result of such treatment. In considering the nature of this hypoglycaemia, the ganglion blocking action of iproniazid(3) may have resulted in a type of hypoglycaemic insensitivity due to inhibition of sympathetic discharge to the adrenal medulla and liver, such as follows hexamethonium therapy(4); or secondly, the slight galactose intolerance noted by Weiss et al. may have been a factor in production of hypoglycaemia, since it is known that an increased blood galactose may have some hypoglycaemic action in the normal individual(5). To try to elucidate these points it was decided to compare the response of total blood sugar and blood glucose levels to a mixed oral dose of glucose and galactose under the influence of ganglion blockade from mecamylamine, and mono-amine oxidase inhibition from iproniazid and nialamide respectively. The latter, unlike iproniazid, produces only minimal postural hypotension(6).

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Methods. The following carbohydrate tolerance test was performed in all subjects. A fasting venous blood sample was collected, then a mixture of 25 g glucose and 25 g galactose in 250 ml water was given by mouth. Further venous samples were withdrawn 10, 20, 30, and 45 minutes later. Blood glucose levels were estimated by a modification of the glucose oxidase method of Middleton and Griffiths(7), and total blood sugar by a copper-reducing method(8).

Fifteen normal adults were studied initially, followed by 12 patients with angina pectoris, half of whom were to be treated with iproniazid 50mg daily, and the other half with nialamide 100mg daily. Carbohydrate tolerance tests were carried out before and 6 weeks after commencing therapy. These patients were asked to record the number of attacks of angina pectoris occurring each day and to note their severity. Finally 3 hypertensive patients were studied before treatment, and again after a postural fall of blood pressure had been obtained following 5 days therapy with mecamylamine 2.5mg twice daily. The tests were carried out 2 hours after the morning dose of 2.5 mg mecamylamine.

Results. The total blood sugar curve in all patients was within normal limits, both before and during treatment. It is seen from Fig. 1 which compares carbohydrate tolerance in a patient before and during treatment with mecamylamine, that while ganglion blockade does not effect the fasting sugar levels, it produces a temporary fall of blood glucose in the early phase of the test. These changes are best summarised in the form of a glucose index (G.I.) which expresses the ratio between blood glucose and total blood sugar (Fig. 2) as follows: Blood glucose (mg per 100 ml) × 100/Total blood sugar (mg per 100 ml) = G. I. In the 15 normal adults, the mean G. I. ± standard deviation was determined. The mean fasting G. I. of the different normal individuals varied from 74.5% to 90.8%, and during the 45 minutes of the test did not change significantly (i.e., blood glucose and total blood sugar levels rose in about the same proportion), although there was an increase in the standard deviation—thus after 45 minutes the normal G. I. range was 66.6-98% (Fig. 2). In some of the angina patients, the amine oxidase inhibitors caused a fall of both fasting blood glucose, and also of glucose levels throughout the period of the test. As total blood sugar remained unaffected, there has evidently been a change in distribution of the blood sugar fractions, which is reflected in a reduced G. I. (Fig. 3 and 4). It is noticeable that the changes resulting from iproniazid are more marked than those from nialamide.

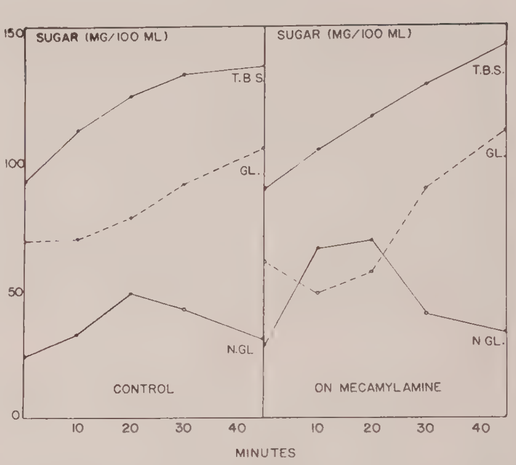
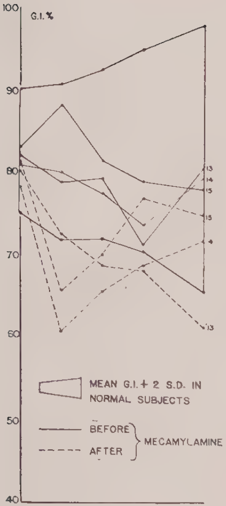


FIG. 1. Effect of mecamylamine on carbohydrate tolerance. T.B.S.—total blood sugar; GL. = glucose; N.GL. = non-glucose sugar by difference.

Eleven of the 12 angina patients claimed some improvement of their symptoms, but analysis of the records showed that the number of attacks per day was halved in only 8 (cases 1-4 on iproniazid, and cases 7-10 on nialamide), (Fig. 3 and 4). All these patients



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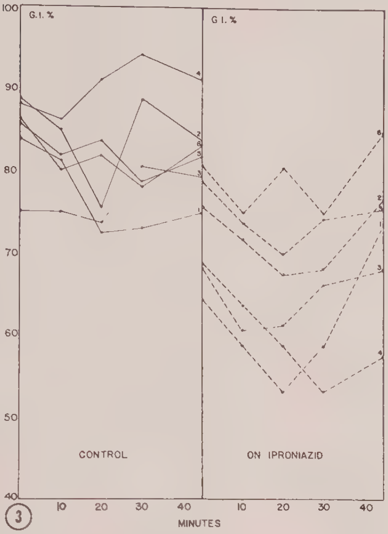

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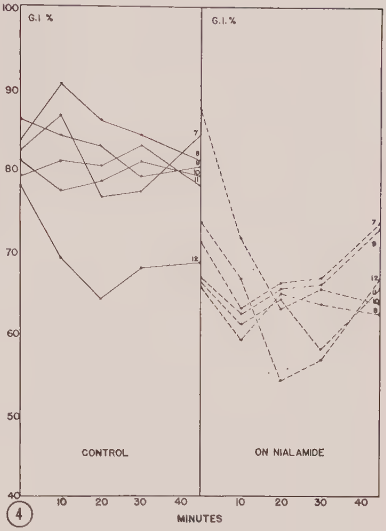

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FIG. 2. Mean glucose index + 2 standard deviations in 15 normal individuals, compared with glucose index in 3 hypertensive patients before and while receiving mecamylamine. Numbers indicate individual patients.

FIG. 3. Glucose index on 6 patients before and after receiving iproniazid for 6 weeks. Numbers indicate individual patients.

FIG. 4. Glucose index in 6 patients before and after receiving nialamide for 6 weeks. Numbers indicate individual patients.

had a fairly consistent fall of the glucose index, both fasting and throughout the test. It is interesting to note that case 4, who showed the greatest clinical improvement (no further chest pain on exertion, and a return to work after 7 years absence on account of chest pain) had the most marked fall of glucose index. By contrast, case 12, who gained no improvement from treatment, and claimed the tablets made him worse, showed an unusually low G. I., before treatment which was unaffected by therapy.

Discussion. The different response to the carbohydrate tolerance test of subjects receiving iproniazid or nialamide on the one hand, and mecamylamine on the other, makes it unlikely that these drugs have a common action on carbohydrate metabolism. Ganglion blockade appears temporarily to enhance the hypoglycaemic action of galactose, probably by a mechanism of decreased hypoglycaemic sensitivity. However, in the case of the mono-amine oxidase inhibitors, the reduced fasting blood glucose, and the lower glucose index throughout the test suggest some other explanation, for example, an interference with hepatic glycogenolysis.

The mode of action of the mono-amine oxidase inhibitors in alleviating the symptoms of angina pectoris is ill-understood. Several alternative hypotheses have been suggested and have recently been reviewed(9). However, in view of this change in carbohydrate tolerance, which appears to coincide roughly with an improvement in symptoms, it seems pertinent to refer to the recently reported improvement in anginal symptoms during treatment with tolbutamide(10, 11). By their action on carbohydrate metabolism both mono-amine oxidase inhibitors and tolbutamide may alter the primary sources of energy utilized by the heart muscle, and thereby modify the symptoms of angina pectoris.

Summary. Twenty-five grams of glucose and 25 g of galactose was given by mouth to 15 normal subjects and to 15 patients. Total blood sugar and blood glucose was estimated on each subject and the glucose index calculated. The test was repeated in the 15 patients after treatment with mecamylamine, iproniazid, and nialamide. The difference in response to ganglion blockade and inhibition of mono-amine oxidase is discussed. The reduction of the glucose index, in this preliminary study, correlated approximately with an improvement in the symptoms of angina pectoris. The possible significance of this is discussed.

Addendum. Since completing these carbohydrate tolerance studies, we have tested the purity of several samples of the standard preparations of glucose and galactose, such as are routinely used in oral glucose and galactose tolerance tests. The galactose preparations had a non-galactose content of 4-14% (w/w), of which about half was copper reducing, and a small fraction glucose oxidase reducing; the glucose preparations had a non-glucose content of 2-10%, most of which was copper reducing, but not glucose oxidase reducing. However, since we used glucose and galactose from the same stock bottles throughout the tests, we believe that the changes of carbohydrate tolerance are genuine.

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Urinary Metabolites of Mevalonic Acid: Evidence That Mevalonic Acid is not a Precursor of Isovalthine.* (27008)

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The discovery(1, 2), isolation(1, 2), identification(2), and synthesis(2) of a new amino acid, S-(isopropyl-carboxymethyl)-cysteine, termed isovalthine has been recently described by Japanese investigators. This amino acid was observed in the urine of hypercholesterolemic individuals. The structure of the amino acid is such as to suggest that it arises biogenetically by addition of cysteine to $\triangle^1$-isopentenyl pyrophosphate followed by or simultaneously with loss of pyrophosphate and oxidation of the primary alcohol to an acid. $\triangle^1$-Isopentenyl pyrophosphate may be visualized as a compound in equilibrium with $\triangle^2$-isopentenyl pyrophosphate which, in turn, is in equilibrium with $\triangle^3$-isopentenyl pyrophosphate. The latter 2 compounds are known intermediates in utilization of mevalonic acid (MVA) and are interconvertible by the enzyme isopentenyl pyrophosphate isomerase(3). If this or a related mechanism has any merit, it would suggest that isovalthine may be a significant "detoxification" product of intermediates in sterol biosynthesis whereby these intermediates are diverted from sterol biosynthesis and appear in the urine as an innocuous metabolite. The experiments described here involving recovery of added carrier isovalthine from the urine of hypercholesterolemic individuals that had received MVA-2-C^{14} demonstrate that either (a) MVA is not a precursor of isovalthine, or (b) MVA may be a precursor of isovalthine but the metabolite is not encountered in all cases of hypercholesterolemia. Evidence is also presented that MVA is the precursor of an amphoteric metabolite possibly related to

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isovalthine.

Materials and methods. DL MVA-2-C^{14} as the N, N′-dibenzylethylenediamine salt (2.48 mc per mM) was administered intravenously to 2 hypercholesterolemic individuals. One subject (HL, F, 58 yrs, hypertensive vascular disease) received 13μc. The other subject (ES, F, 47 yrs., coronary artery disease) received 20μc. At the time that the 2 subjects were studied one (HL) had a total cholesterol blood level of 301 mg per 100 ml while the other (ES) had a level of 371 mg per 100 ml. Neither subject was receiving antihypercholesterolemic therapy at the time these studies were done. Complete 24-hour urine collections from each subject were obtained following MVA administration and these collections were then frozen until studied in the laboratory.

Isovalthine at a level of 1 mg per ml was added to each urine sample immediately after unfreezing. In the case of HL this amounted to 1420 mg; in the case of ES, to 1200 mg. The isovalthine used was synthesized by condensation of L-cysteine with DL-α-bromoisovaleric acid in aqueous alkali according to a previously published procedure(4). The product was, therefore, a diastereoisomeric mixture. It has not, as yet, however, been possible to separate the diastereoisomers by fractional crystallization. Each urine sample was then poured onto a column of 150 ml of Dowex-1 freshly prepared in the hydroxide form. The urine was allowed to run through the column at a rate of about 10 ml per min and was followed with several hundred ml of water. The column was then eluted with 0.1 N hydrochloric acid. One hundred ml fractions of the run through and eluates were collected, and 0.01 ml amounts of the various fractions were spotted on a sheet of Whatman #1 paper and developed by ascending chromatography in butanol: acetic acid: water (160: 40: 40). The added isovalthine was located with ninhydrin (RF of about 0.53). Two ml amounts of the various fractions were also pipetted into planchets (2 inch diameter, stainless steel, concentric rings), neutralized where necessary to phenolphthalein and evaporated to dryness in a stream of air at room temperature. The samples were counted in a thin window gas flow counter. Those eluate fractions containing the isovalthine which coincided with those fractions containing radioactivity were combined.

The combined fractions from the Dowex-1 column were poured onto a column of 200 ml of Dowex-50 freshly prepared in the acid form. The column was washed with several hundred ml of water and then eluted with 2 N ammonia. 0.01 ml amounts of the various fractions (100 ml) were spotted on a strip of Whatman #1 paper and the isovalthine located with ninhydrin. Two ml amounts of the various fractions were also counted as in the previous step. Those fractions containing the isovalthine were combined and concentrated *in vacuo* to a small volume in a rotary evaporator.

The concentrated fractions from the Dowex-50 column were poured on to a column of 150 ml of Dowex-1 freshly prepared in the acetate form. The column was washed with several hundred ml of water and then eluted with 2 N acetic acid. The isovalthine was located and radioactivity of the various fractions determined as described earlier. Those fractions containing the isovalthine were evaporated *in vacuo* to dryness in the rotary evaporator.

The isovalthine fractions from the previous step were dissolved in a small volume of water and treated at steam bath temperature with norite. The filtrate from the norite treatment was concentrated to a small volume and ethanol added to a concentration of about 50%. Isovalthine crystallized on cooling and was filtered off, washed with 50% ethanol, and dried at room temperature. Identity and homogeneity of the products was established by paper chromatography and infrared spectrum analysis.

The recovered isovalthine was examined for radioactivity by liquid scintillation counting in a Packard 'Tri-Carb' scintillation spectrometer. This method of counting is approximately 4 times more sensitive than the gas flow counting used with the various chromatographic fractions. The mixtures counted contained 20 mg of recovered isovalthine, 1 ml of 1 M 'Hyamine' solution in methanol and 10 ml of scintillation mixture (4 g PPO and 30 mg POPOP per liter of toluene.)

Results. The results obtained are summar-

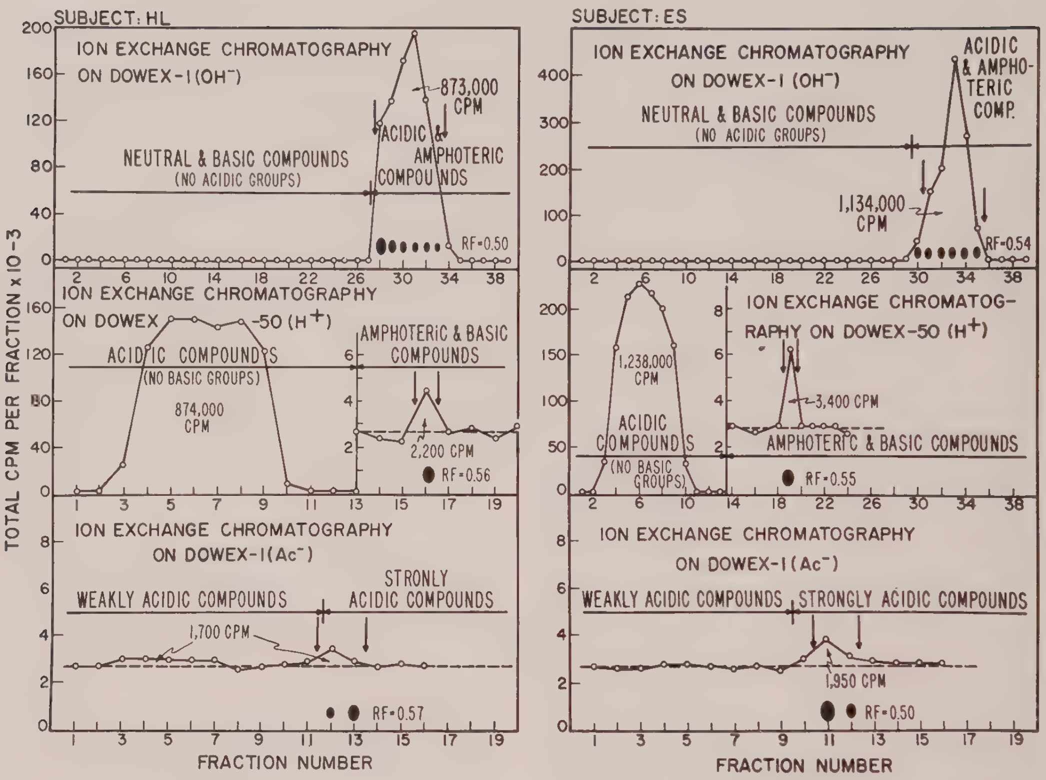

FIG. 1. Summary of chromatographic studies. Those fractions of a chromatogram that were combined and subjected to subsequent chromatography or crystallization are indicated between the vertical arrows. Counts summarized are *total* counts per fraction. Background where significant is indicated by a dashed line. The darkened elipses indicate location and approximate amount of added isovalthine as determined separately by ascending paper chromatography in butanol: acetic acid: water (160: 40: 40) and development with ninhydrin.

TABLE I. Summary of Information on Recovered Isovalthine.

Exp	Material studied	Radioactivity* counts in 30 min	RF value†
HL	Recovered isovalthine	577	.51
	Reference "	579	.49
ES	Recovered isovalthine	573	.51
	Reference "	572	.48

* Each sample counted contained 20 mg of recovered or reference isovalthine, 1 ml 'Hyamine,' and 10 ml of scintillation mixture.

† Ascending chromatography in butanol: acetic acid: water (160: 40: 40).

ized in Fig. 1 and in Table I. When urine from hypercholesterolemic individuals administered DL MVA-2-C^{14} and to which non-radioactive isovalthine had been added is chromatographed on the strong anionic exchange resin Dowex-1 in the hydroxide form radioactive material and isovalthine are retained by the column and are eluted with dilute hydrochloric acid (Fig. 1). The radioactive material is without doubt largely unnatural MVA (from the racemate administered) which is known to be inactive biochemically(5). When the hydrochloric acid eluates are chromatographed on the strong cationic exchange resin Dowex-50 most of the radioactivity of the urine is not retained by this resin as would be expected if the radioactivity is largely unnatural MVA (no basic groups). On the other hand, a small fraction of the radioactivity of the original urine is retained and since it had been previously retained by an anionic exchange resin it must represent amphoteric material. This radioactivity coincided with the isovalthine but it should be appreciated that no fractionation of amphoteric material is accomplished with the strong eluting agent (2 N ammonia) employed. When the ammonia eluates are chromatographed on the strong anionic exchange resin Dowex-1 in the acetate form most of the radioactivity is retained by this resin and is eluted by acetic acid (2 N). Under the conditions of the chromatography employed, monoamino monocarboxylic amino acids are not retained by the resin but monoamino dicarboxylic amino acids are held and eluted with the acetic acid. The results obtained furnish presumptive evidence that the radioactive, amphoteric metabolite in the urine of hypercholesterolemic individuals administered MVA-2-C^{14} is a monoamino dicarboxylic acid. On the other hand, isovalthine recovered from the radioactive fractions of the Dowex-1 acetate column is devoid of radioactivity (Table I). These results form the basis for the conclusion that either (a) MVA is not a precursor of isovalthine, or (b) MVA may be a precursor of isovalthine but the metabolite is not encountered in all cases of hypercholesterolemia.

Discussion. These studies have furnished evidence that MVA does not serve as a precursor of isovalthine in the hypercholesterolemic individual but instead is converted in small amounts into some other amphoteric metabolite presumably a monoamino dicarboxylic acid. The unqualified conclusion that MVA is not a precursor of isovalthine is not justified because of a possible alternative interpretation of the data. Obviously if isovalthine is derived from MVA but is only randomly encountered in the urine of hypercholesterolemics it is possible that the amino acid was not initially present in the urine samples examined. Under this condition carrier isovalthine would have been recovered without radioactivity following administration of radioactivity while in similarly treated hypercholesterolemics excreting isovalthine carrier isovalthine would have been recovered with radioactivity. If this is the explanation for the failure to recover radioactivity in carrier isovalthine then these studies have significance in that they show that isovalthine is not a ubiquitous, quantitatively significant metabolite of sterol intermediates.

If MVA is not a precursor of isovalthine, an explanation seems necessary for the existence in urine under conditions of what is probably increased sterol biosynthesis (hypercholesterolemia) of a metabolite that does not originate from sterol intermediates. Possibly isovalthine originates from a sterol precursor before MVA in the biosynthetic sequence. More likely under conditions of excess sterol biosynthesis relatively non-specific mechanisms for the diversion of sterol intermediates away from the "normal" course come into play and various compounds related in structure to sterol intermediates are indiscriminately metabolized.

Summary. Synthetic isovalthine (S-(isopropyl-carboxymethyl)-cysteine) was added to urine from hypercholesterolemic patients that had received mevalonic acid-2-C^{14} intravenously. Isovalthine recovered from the urine was found to be devoid of radioactivity thus indicating that either (a) mevalonic acid is not a precursor of isovalthine, or (b) mevalonic acid may be a precursor of isovalthine but the metabolite is not encountered in all cases of hypercholesterolemia. The existence in the urine samples studied of an amphoteric metabolite of mevalonic acid was demonstrated.

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Irritative Deposits from Stainless Steel Electrodes in the Preoptic Rat Brain Causing Release of Pituitary Gonadotropin.* (27009)

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Recently developed methods have made possible artificial induction of ovulation in female rats in which spontaneous ovulation has been blocked by certain drugs, notably pentobarbital. In rats exposed to controlled illumination 14 hours daily, the ovulation-inducing hormone is normally released from the adenohypophysis sometime between 2:00 and 4:00 P.M. on the afternoon of proestrus(1). The neurogenous stimulus which brings this about can predictably be blocked by suitably timed injection of pentobarbital(2). If, during the course of anesthesia, such an animal is subjected to electrical stimulation of the brain through electrodes resting in the tuber cinereum immediately over the median eminence, ovulation follows during the ensuing night(3). The procedure has also been effective in constant-estrous rats(4). Studies carried out in this laboratory(5) have shown that for inducing ovulation the preoptic region is at least as effective a stimulation site as the tuber cinereum. In fact, with currents of the characteristics that we have employed as standards, results with preoptic electrodes have been considerably more consistent than with electrodes close to the median eminence. In the preoptic region there is a greater latitude with respect to electrode position, suggesting that at this level the pathway in question is diffuse and that it becomes more restricted as it moves caudally into the tuber cinereum. Such an interpretation is in agreement with earlier observations by Hillarp(6).

The ovulation-inducing effect of preoptic stimulation is not blocked by atropine in dosage that will regularly block spontaneous ovulation(7), indicating that, like pentobarbital, atropine blocks the spontaneous event without involving the hypothalamico-hypophyseal system. When first encountered, the observation presented a paradox, for in earlier investigations(1,8) it had been concluded that the atropine-sensitive phase of the process leading to release of ovulating hormone lasts for about half an hour, concurrently with the discharge of hormone from the hypophysis. Complete discharge seemed dependent on continuous action of the atropine-sensitive mechanism throughout this time. Yet the electrical stimulation data disclosed that, under pentobarbital or atropine blockade, ovulation can be produced by a train of pulses lasting only 60 seconds, a clear suggestion of a triggering action. The observations recorded below, however, supply a reasonable explanation of the discrepancy by showing that, although the stimulus is introduced by electric current, the current produces an irritative focus which continues to act long after the electrode is withdrawn.

Methods. All animals used were adult females of our inbred stock. All had a vaginal smear history of at least two 4-day cycles in sequence and were again in vaginal proestrus on the day of experiment. Pentobarbital was injected intraperitoneally a few minutes before 2:00 P.M., the dose being 35 mg/kg body weight, an amount known to be uniformly effective in blocking spontaneous release of ovulating hormone. During the anesthesia, each animal was placed in the stereotaxic instrument with the aid of brief supplementary anesthesia with ether.

Electrodes were of several types. The standard one was a concentric, bipolar, stainless steel unit constructed of a 0.4 mm external diameter tube containing a Teflon-insulated 0.15 mm core. The unit was insulated externally with Formvar† or Epoxylite‡, both ele-

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† Formvar 15/95E, Shawinigan Resins Corp., Springfield, Mass.

‡ Epoxylite 6001-M Electrode Insulator, Epoxylite Corp., El Monte, Calif.

ments being bared at the tips. The core extended about 0.5 mm beyond the end of the tube. Characteristically, the core was the anode. For special purposes indicated later, concentric units of similar dimensions were constructed of platinum tubing and wire. For other special purposes parallel bipolar units were constructed of either reagent-grade iron wire# (analysis, 99.8% Fe; diam., 0.22 mm) or platinum wire (0.25 mm). For stiffness, one wire (the anode) was sheathed in glass and the other was insulated and cemented to the outside of the glass by dipping the assembly in Formvar repeatedly. The 2 wires were bare at the tips, with the cathode 1 mm shorter than the anode. In practice, this type of electrode was placed with the anode in the preoptic region and the cathode turned anteriorly.

Electric current was derived from a Grass S-4 stimulator and SIU-4 isolation unit. Although a wide variety of electrical parameters has been used, the following are standard: monophasic, rectangular 1 msec pulses, 100/sec, 100 μA, continuously for 60 sec or in trains of 30 sec at 30 sec intervals for 5 min. The current was routinely monitored by a cathode ray oscilloscope across a resistor in series with the preparation. The oscilloscope was calibrated at time of experiment with a precision microammeter (Simpson Model 269 VOM). Additional resistance of approximately 400 KΩ was introduced in the leads to the preparation to minimize the effect of electrode capacitance on the wave form and thus to produce an essentially rectangular wave on the monitor screen. The current in microamperes could then quickly be read from the height of the pattern. The relatively high *open-circuit* voltage of such a system is immaterial to the results described, as demonstrated by the fact that 0.05 msec pulses produce neither ovulation nor the characteristic histologic reaction described below. Conversely, both effects are readily obtained in circuits that lack the high peripheral resistance and hence utilize correspondingly low open-circuit voltage.

For experiments requiring introduction of minute volumes of fluid into the brain, the injection was accomplished by means of a tuberculin syringe driven by a machine screw. A length of polyethylene tubing (Clay-Adams PE-50) led from the syringe to a glass cannula 0.4-0.5 mm in diameter and 6-8 cm long, attached to the electrode carrier of the stereotaxic apparatus. The system was filled with mineral oil lightly colored with Oil Red O, after which the aqueous solution intended for injection was drawn far into the cannula. Delivery of the desired volume of fluid was measured directly by excursion of the end of the oil column between calibration marks.

The results of all experiments were determined the next morning by direct observation of presence or absence of eggs in the Fallopian tubes(2). In seemingly negative cases the ovaries were preserved for histologic examination. The brain was fixed by perfusion with 10% formalin in physiological saline and subsequent immersion in the mixture for at least 24 hr. Blocks containing tracks of electrodes or cannulae were serially sectioned at 25μ and stained in Luxol Fast Blue—Cresyl Fast Violet(9). In special cases, for the Prussian Blue test, 1% potassium ferrocyanide was added to the perfusion medium and the brain sections were counterstained with Darrow Red(10).

Tissue Reaction to Stimulating Current. Histologic study of the brains from the many animals subjected to electric currents of widely varied parameters disclosed that in all cases of ovulation there was a zone of electrolytic damage about the electrode tips and that in negative cases the area affected tended to be significantly smaller if recognizable at all. Characteristically, in the damaged areas (Figs. 1 and 2) outright coagulation was restricted to a small spot immediately at the end of the electrode track. Outside of this, there was a more extensive zone of mild inflammation in which few nerve cells were to be found; some nerve fibers could still be recognized but their myelin staining was greatly diminished; neutrophilic leukocytes were scattered in small numbers throughout. In 43 representative brains, tracings of the affected areas were made by projection and their diameters were measured perpendicular to the electrode track. The marked difference between positive and negative cases can easily be seen in Table 1. Only 3 of the 16 negative specimens had lesion diameters significantly larger than the elec-

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# We are indebted to Prof. Douglas G. Hill, Chemistry Dept., Duke University, for a generous sample of this material.

trode track itself. Size of the lesion was, in general, correlated with amount of electricity delivered, expressed in coulombs.

TABLE I. Relation Between Amount of Electrolytic Damage of Preoptic Tissue and Induced Release of Pituitary Ovulating Hormone.

Diam. of lesion (mm)	Experimental results		
	Ovulation	Partial* effect	Negative
<.3‡	0	3	13
.3-.39	4	2	1
.4-.49	0	0	2
.5-.59	10	2	0
.6-.69	5	0	0
.7-.79	1	0	0

* Fewer than 7 ova in Fallopian tubes.

‡ Minimal, not significantly larger than electrode track.

Effect of Direct Current. Because of the monophasic character of the pulses, it seemed possible that we were dealing with an effect of metals secondary to erosion of the anode. In a small series of experiments, in fact, the Prussian Blue test clearly demonstrated the presence of iron at the end of the electrode track.

This fact, coupled with the relation of result to size of lesion and quantity of electricity, called for a test of direct current. This was immediately successful. Among 14 rats, currents ranging from 150 μA to as little as 10 μA for as short a time as 20 sec gave uniformly positive results. Even 15 μA for 10 sec gave full ovulation in 5 of 6 rats and partially ovulated the other (6 tubal ova, 4 unruptured follicles). By contrast, 7.5 μA for 10 seconds failed to ovulate any of the 5 rats thus treated.

Results with Platinum Electrodes. It has been reported that in contrast to stainless steel electrodes, electrodes of platinum-iridium are not subject to erosion when imbedded in brain tissue(11). In an attempt to avoid the effects noted above, concentric electrodes were prepared from platinum (see Methods). Eleven rats were stimulated with this type of electrode placed in the preoptic region. The characteristics of the stimulating current in each case were such that ovulation would have been induced in essentially every rat had stainless steel electrodes been used. Only 1 rat ovulated completely, 1 rat had a single ovum, and 9 rats were negative. (Table 2).

TABLE II. Attempted Induction of Ovulation with Platinum Electrodes.

Stimulus: rectangular, monophasic, 1 msec pulses, 100/sec Current (μA)	Time	Results		
		Ovulation	Partial effect	Negative
150	5 min*	0	1	1
100	5 " *	1	0	5
100	60 sec	0	0	3

* 30 sec "on," 30 sec "off" each min.

Microinjection of FeCl$_3$. By means of the microsyringe, with the end of the glass cannula resting in the preoptic region, small amounts of 5% aqueous FeCl$_3$ were injected into the brain substance (Table 3). In control animals, like amounts of 5% NaCl were injected, with pH adjusted in most cases to 1.3 to correspond with the pH of the FeCl$_3$. Ovulation was induced in all rats that received 1 μl FeCl$_3$ but it occurred in only one of the 8 rats that received acidified NaCl. Histologic study disclosed a pronounced inflammatory reaction to FeCl$_3$ in areas measuring 3-4 mm in diameter in the case of 1 μl injections and 1-1.5 mm in the rats that received 0.1 μl. There was comparatively much less reaction to NaCl, but it was nevertheless significant, especially when NaCl was acidified. Diameters of the affected zones were between 0.5 and 1 mm.

TABLE III. Microinjections into Preoptic Tissue.

Treatment	Results	
	Ovulation	Negative
1. μl 5% FeCl$_3$	8	0
.1 " " "	2	2
1. μl 5% NaCl, pH 5.3	0	4
1. " " " pH 1.3	1	7

Electrodes of "Reagent" Iron vs. Platinum. The electrodes employed were the parallel bipolar units made of reagent iron or platinum wires. Eight proestrous, pentobarbital-blocked rats were subjected to 100 μA D.C. for 30 sec through the iron wire, and 7 rats were subjected to 500 μA D.C. for 30 sec through platinum. Seven of the former rats ovulated and all of the latter were negative. The lesioned areas were about equal in size; the larger amount of current was used in the platinum experiments to insure this. But while the iron electrodes produced the familiar zone of inflammation with minimal coagulation (Fig. 3), the chief effect of the platinum electrodes was to coagulate (Fig. 4) and there was hardly any tissue reaction outside the coagulum.

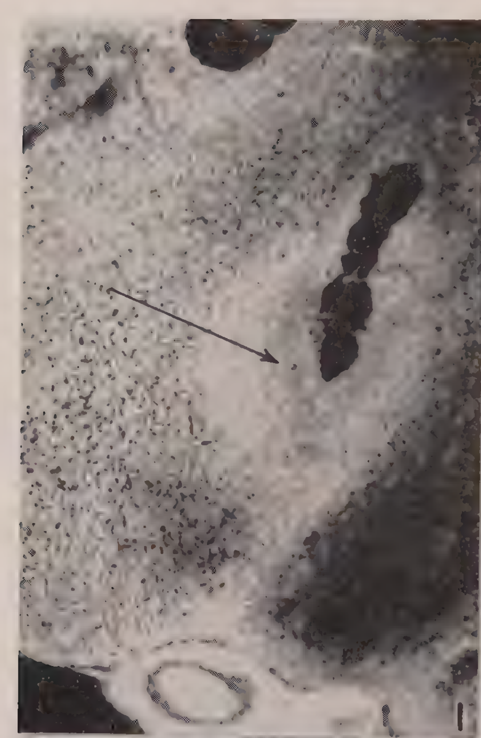
Discussion. Hillarp and associates(12) described prolonged behavioral after-effects of bilateral electrolytic lesions placed basally in the lateral preoptic region of the rat brain. Beginning 20 to 30 minutes after recovery from ether anesthesia, rats of either sex developed a strong drive to male-type copulatory behavior, which lasted several hours. The electrode material was nichrome, another iron-containing alloy. Electrodes of nichrome may be effectively substituted for stainless steel in preoptic excitation of the ovulation mechanism (Riley and Everett, unpublished). It is reasonable to assume that both the behavioral effect observed by Hillarp and the ovulation phenomenon described here result from *irritative* action of local deposits of metallic compounds, perhaps not only of iron but of other metals as well. We conclude that in both circumstances the irritation continues long after withdrawal of the electrode, furnishing a reasonable solution to the paradox earlier mentioned in relation to the atropine blocking experiments.

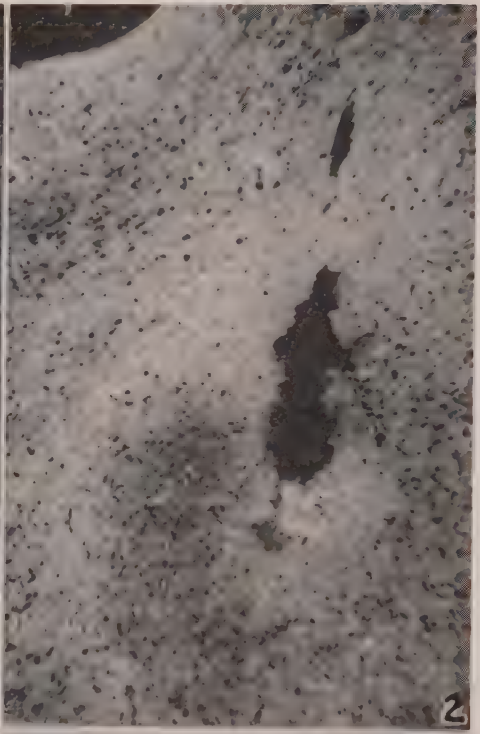
The data suggest that preoptic neurons which control release of ovulating hormone, while easily subject to this grossly unphysiological type of excitation, are difficult to excite by more strictly electrical stimuli. Other possible interpretations should be considered, however. It may be that electrical stimulation (i.e., through platinum electrodes) should continue throughout the time needed by the pituitary gland to discharge the amount of hormone necessary for ovulation. Hypophysectomy experiments, at varying intervals after electrolytic stimulation of the brain, have disclosed that such hormone release requires between 30 and 40 minutes under ether anesthesia and between 45 and 60 minutes under pentobarbital(13). The optimal pulse form and frequency may differ from those tested. Perhaps the virtue of the electrolytic stimulus is that its irritative after-effect extends radially through a considerable volume of brain tissue, stimulating fibers throughout the damaged zone or at its periphery; pulses administered through platinum electrodes, as presently designed, may actuate relatively few fibers.

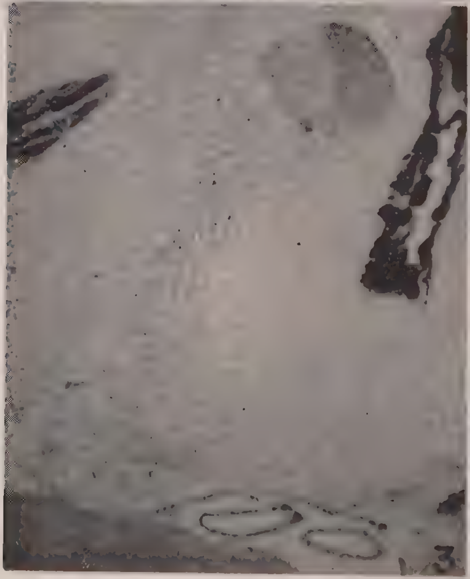
In causing the discharge of ovulating hormone under pentobarbital sedation, stimulation by electrolysis has been effective not only in the preoptic region, but somewhat more caudally in the anterior hypothalamus and, in preliminary experiments, more rostrally in the medial parolfactory area, in parts of the nucleus accumbens, and in septal nuclei dorsal and anterior to the anterior commissure. Anatomical and physiological characterization of the neurons involved in the phenomenon will be objectives of future study. It is appropriate to note here that consistent failure has resulted from our attempts to provoke ovulation by (unilateral) electrolytic stimulation of tuberal regions, using small currents that would be uniformly effective in more rostral locations, and that although the mere insertion of an electrode into the hypothalamus has been reported to release enough LH to be detected by the ovarian ascorbic acid depletion test(14), this is clearly not adequate to produce significant luteinization under the conditions of our experiments.

Finally, the present findings urge great caution in use of electrodes of stainless steel or nichrome for stimulation. In reporting either stimulation experiments or the short-term effects of electrolytic lesions, not only should the electrical parameters be specified, but the physical and chemical characteristics of the electrode itself should be described.

Summary. When ovulation was induced by means of stainless steel electrodes in the preoptic area, delivering pulses of varied characteristics, a prominent zone of mild inflammation appeared at the end of the electrode track. When ovulation failed, the zone was characteristically small or absent. Electrolysis with direct current, 10 μA or more for 20 sec or longer, proved uniformly effective in causing ovulation. Substitution of platinum electrodes for stainless steel usually gave negative results. Microinjection of $FeCl_3$ solution into the preoptic tissue induced ovulation, while injection of similar amounts of acidified NaCl did not. Finally, electrolysis stimulated ovulation when electrodes of reagent grade iron wire were used, but not when similar electrodes of platinum were employed. It is concluded that iron and perhaps other metals electrolytically deposited from electrodes of stainless







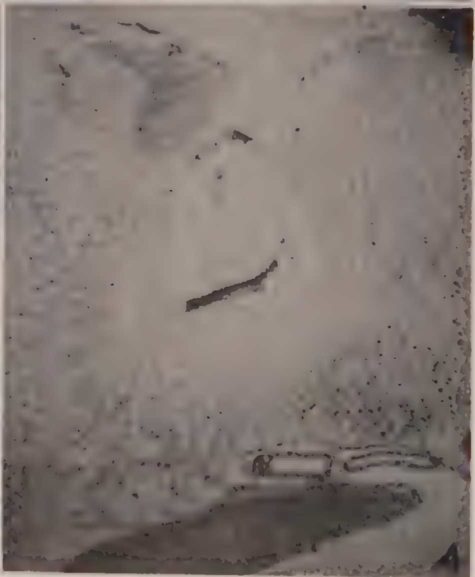


FIG. 1. Parasagittal section of preoptic area (right) and anterior hypothalamic area (left) in brain of an experimental rat, showing tissue reaction (arrow) about the end of the electrode track. Concentric stainless steel electrode. Ovulation induced by monophasic, rectangular pulses, 1 msec duration, 100/sec, 100 μA, 60 sec. Anterior commissure above, optic chiasma lower left, diagonal band to right and somewhat down. Luxol Fast Blue—Cresyl Fast Violet. 50 X.

FIG. 2. Higher magnification of a similar zone of electrolysis. 100 X.

FIG. 3. Electrolytic effect of anodic direct current (100 μA, 30 sec) delivered by an electrode of reagent iron. Ovulation induced. 35 X.

FIG. 4. Electrolytic effect of anodic direct current (500 μA, 30 sec) delivered by an electrode of platinum. No ovulation 35 X.

steel (or nichrome) produce irritative foci whose stimulative action on certain neurons continues long after the flow of electric current has ceased.

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Effect of Cold Acclimation on Vitamin A Metabolism.[*] (27010)

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Rodahl and Moore(1) reported that the liver of the polar bear contains a very large amount of vit. A, and concluded that the toxic effects noted in man following his ingestion of polar bear liver are symptoms of hypervitaminosis A. They also mentioned that similar findings are sometimes seen with the liver of seals.

It would seem most likely that this high hepatic Vit. A content of polar bear and seal livers is related to the nature of the diet of these animals. However, it is also possible that low environmental temperature may play a direct role. To test this possibility, experiments designed to determine the effect of cold acclimation on hepatic vit. A content were carried out with rats.

Experimental. Adult male rats of the Wistar strain were fed *ad libitum* for 10 days a standard diet of the following composition: 25% casein, 10% lard, 51% dextrose, 3% salt mix (2), 5% cellulose, 6% brewers yeast, 0.04% cod liver oil concentrate, 0.01% alpha-tocopherol. After a period of dietary stabilization, the rats were divided into the following 4 groups: Group A was fed the standard diet *ad libitum* for 4 to 5 months while living at 25°C; Group B was fed the standard diet *ad libitum* for 4 to 7 months while living at 0−2°C; Group C was fed the standard diet minus the cod liver oil concentrate *ad libitum* for 4 months while living at 25°C; Group D was fed the standard diet minus the cod liver oil concentrate *ad libitum* for 4 months while living at 0−2°C. In the case of Groups A and B the diets contained 34 units of vit. A per gram of diet, thus providing an abundance of the vitamin. In the case of Groups C and D the rats were administered orally 40 units of vit. A as cod liver oil concentrate in carrier corn oil once a week, thus providing just over the minimum vit. A requirement for rats(3).

At the end of the experimental period the rats were killed by decapitation. The liver was excised, weighed, wrapped in aluminum foil and quickly frozen in liquid nitrogen. The livers were stored in the frozen state at −30°C until analysed for vit. A content.

The method of Ames *et al*(4) was used for vit. A analysis. These authors point out that when levels of vit. A are below 10 γ per gram of liver, turbidity problems can interfere with the assay. In many of the livers analysed in the present work, levels of less than 10 γ of vit.

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[*] This work was accomplished under Air Force Contract monitored by Alaskan Air Command, Arctic Aeromedical Lab., and was also aided by Grants from Nat. Inst. Health and from Whitehall Foundation.

A per gram of liver were encountered. However, we found that vigorous centrifugation of suitable aliquots of liver extract overcame the turbidity problem in all but one instance. In one case the turbidity could not be successfully dealt with; the data from this assay were discarded.

Results and Discussion. The data on the effects of cold acclimation on vit. A content of the liver of rats allowed to eat a vit. A containing diet *ad libitum* are presented in Table 1. Livers of cold-acclimated rats contain much more vit. A than do livers of rats maintained at 25°C. Moreover, since the liver of the cold-acclimated rat is smaller than that of the control rat, the increase in vit. A content accompanying cold acclimation is even greater when the data are expressed on a per gram liver basis.

TABLE I. Effects of Cold Acclimation on Hepatic Vit A Content of Rats Fed *Ad Libitum* a Diet Containing Vit A.

Physiological State	No. of Rats Used	Vit A Content of Liver Total γ	γ/g wet wt
Acclimated to 25°C	6	12,230 ± 2066*	1160 ± 73
“ “0-2°C	6	24,400 ± 5660	2950 ± 361

* SEM.

In considering the reasons for this increase in hepatic vit. A content during cold acclimation, it seemed most likely that it resulted from an increase in vit. A intake, a simple corrollary of the increased food consumption observed in cold-acclimated animals(5,6). To test this possibility experiments were carried out in which rats were fed at either 25°C or 0−2°C the standard diet from which the cod liver oil concentrate had been deleted. Each week these rats were given 40 units of vit. A. A dosage of vit. A near the minimum required amount was chosen so that any alteration in vit. A metabolism leading to vit. A sparing in cold-acclimated rats would be exaggerated.

The results of these experiments are recorded in Table 2. When fed the same amount of vit. A, the cold-acclimated rats store a little less vit. A in the liver than do the rats acclimated to 25°C, the p being less than 0.01 when expressed on the basis of total liver and less than 0.02 when expressed on the basis of per gram liver weight. Thus no evidence was found to indicate that cold-acclimation exerts a sparing action on vit. A metabolism.

TABLE II. Effects of Cold Acclimation on Hepatic Vit A Content of Rats Administered 40 Units of Vit A per Week.

Physiological State	No. of Rats Used	Vit A Content of Liver Total γ	γ/g wet wt
Acclimated to 25°C	9	103 ± 10.4*	10.2 ± 1.13
“ “0-2°C	10	57 ± 7.8	6.5 ± .85

* SEM.

These data appear to support the generalization that vit. A requirement is not related to food intake, i.e., to energy metabolism, but is more closely correlated with body size(7). The fact that this holds even when the voluntary food intake is increased by cold acclimation is an interesting observation.

Summary. Hepatic vit. A levels are much higher in cold-acclimated rats than in rats maintained at 25°C. This increase in hepatic vit. A content does not stem from a sparing action induced by cold acclimation but is rather a simple corollary of increased food consumption that accompanies cold-exposure. Moreover, evidence is presented which suggests that the amount of vit. A needed for rats to live at 0-2°C is not markedly greater than the amount needed to live at 25°C.

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Mechanism of Aminonucleoside-Induced Nephrosis in the Rat. II. Metabolism of Aminonucleoside-8-C^{14}.* (27011)

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Study of the metabolism of tritiated aminonucleoside, 6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)-purine, has failed to provide evidence for the conversion of this nephrotogenic agent into molecular structures closely resembling those of the adenosine-containing nucleosides, nucleotides or cofactors(1). Certain other observations(2,3), however, have raised a number of questions concerning the validity of these findings. Littlefield and Dunn (2), for example, have reported the presence in trace amounts of both the 6-dimethylamino- and the 6-methylamino-purine in rat liver microsomal ribonucleic acid; and, Wilson *et al*(3) were able, in a dialyzed yeast enzyme system, to demonstrate nucleotide formation from 6-amino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)-purine and the 6-methylamino and 6-dimethylamino derivatives thereof. Rate of conversion of the latter to nucleotide, however, was only 1/10th that of the 6-methylamino derivitive and only 6/100th that of the 6-amino compound(3).

Considering these observations in conjunction with the possibility that the sensitivity of our tracer experiments may have been seriously impaired by labilization of tritium from the labeled aminonucleoside and subsequent exchange of the isotope with the hydrogens of body water(1), it seemed highly desirable to reinvestigate the metabolism of aminonucleoside using carbon-14 as a tracer in the 8-position of the purine ring. Use of this labeling would not only increase the tracer sensitivity of the experiments through elimination of the loss of labilized tritium, but would also offer the additional advantage of limiting labeled metabolic products to those containing the purine-8-C^{14} moiety. The present report is concerned with the results of such studies and includes, in addition, observations on the chemical nature of carbon-14 activity found in both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), isolated from kidney and liver tissue residues remaining after perchloric acid extraction of the acid-soluble nucleotides.

Materials and Methods. Radiochemical purity of aminonucleoside-8-C^{14}. Establishment of the radiochemical purity of carbon-14 labeled aminonucleoside‡ was accomplished by chromatography of samples of labeled aminonucleoside, unlabeled aminonucleoside, and a mixture of the two on Whatman No. 1 paper, as described by Hotchkiss(4). Following descending development in the solvent system, n-butanol saturated with water, the results of which indicated substances moving with identical R_f values, the chromatogram of the labeled aminonucleoside was cut into 10 mm segments and each segment extracted with distilled water. Ultraviolet absorption at 268 mμ and carbon-14 activity were determined on each extract. Correspondence of carbon-14 activity with ultraviolet absorption and agreement of R_f data were considered indicative of the radiochemical purity of the labeled material.

Measurement of radioactivity. Sample vials were prepared as previously described(1) and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 314. All specific activity data were corrected for quenching effects determined by utilization of an internal standard. The overall counting efficiency for standard C^{14}-labeled benzoic acid in the system was 43.5%.

Types of metabolic experiments. Two types of experiments were conducted. In one of these, the nephrotic syndrome was induced in each of of 3 Sprague-Dawley virgin female rats by essentially the procedure of Fiegelson, Drake, and Recant(5). The rats were maintained in separate metabolism cages throughout the ex-

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periment and fed Rockland rat checkers *ad libitum.* Each rat was injected subcutaneously with 5 mg aminonucleoside-8-C^{14} per day for a period of 10 days. This level of treatment corresponded to approximately 16.5 μmoles of aminonucleoside per day and 1 μcurie of carbon-14 per day. The material was injected in the form of a 2% solution of the nucleoside in isotonic saline. Twenty-four hours after final injection, the rats were sacrificed by ether anesthesia and exsanguination. In the other type of experiment, no attempt was made to induce the nephrotic syndrome. Metabolism of aminonucleoside-8-C^{14} was studied following a single dose of 10 mg of labeled material containing 2.3 μcuries of carbon-14 administered 3 hours prior to sacrifice.

Preparation of acid-soluble nucleotide fraction and isolation of BaRNA and BaDNA. Kidney and liver tissue were immediately removed and either frozen on dry ice and stored at −15°C or processed immediately by the procedure of LePage and Heidelberger(6) for acid-soluble nucleotides and the barium salts of the nucleic acids. In general this procedure involves homogenization of the tissue in cold 4% perchloric acid, separation of the acid-soluble nucleotide fraction by centrifugation, removal of phospholipids from the tissue residue by alcohol-ether extraction, alkaline hydrolysis of the defatted tissue residue permitting separation of ribonucleotides from deoxyribonucleic acid, and finally, precipitation of the latter 2 fractions as the barium (Ba) salts.

Chemical nature of carbon-14 activity in the acid-soluble nucleotide fraction. The 4% perchloric acid extracts containing the acid-soluble nucleotide fraction were cooled in an ice-salt bath and carefully neutralized to pH 6-7 with 5 N KOH using phenol red as an internal indicator. Perchlorate thus precipitated as the potassium salt was removed by centrifugation and the supernatant filtered. The pH of the filtrate was then adjusted to 6 for chromatography on 20 x 1 cm Dowex 1-X10 columns (formate) as described by Hurlbert *et al*(7). Elution of the nucleotide fractions from the column was followed by measuring the 260 mμ absorption in the Beckman DU Spectrophotometer. Carbon-14 activity was determined in each peak and fractions found to contain radioactivity were pooled and concentrated by lyophilization. Residues were then dissolved in water, the pH adjusted to 6, and the resulting solution rechromatographed on 10 x 1 cm Dowex 1-X10 columns with the modified ammonium formate system. Carbon-14 containing peaks obtained in rechromatography were identified by spectral characterization and chromatography on Whatman No. 1 paper with Solvent System III (8).

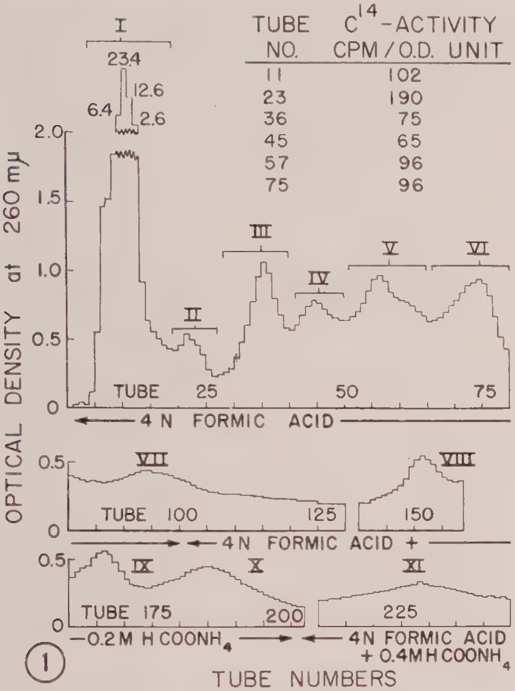
Chemical nature of carbon-14 activity in BaRNA and BaDNA fractions isolated from kidney and liver tissue. Samples ranging from 10 to 50 mg of the barium salts of the ribonucleotides or DNA were dissolved in 1 N HCl and heated for 1 hour in a boiling water bath. Barium ion was then removed by adding a slight excess of sulfuric acid and separation of the barium sulfate precipitate by centrifugation. Hydrolysates thus prepared were placed on 90 x 9 mm Dowex 50-X12 columns and chromatographed as described by LePage and Heidelberger(6). Eluate peaks found to contain carbon-14 activity were identified by position in the eluate, wave lengths of maximum extinction, and 250/260 and 280/260 absorbance ratios.

TABLE I. Distribution of Carbon-14 Activity* in Acid-Soluble Nucleotide and Nucleic Acid Fractions of Kidney and Liver Tissue.

Fraction	Activity	
	Kidney	Liver
	Counts/min/g tissue extracted	
Acid-soluble nucleotide (4% Perchloric acid extract)	17,280	6400
	Counts/min/mg barium salt	
BaRNA	46	129
BaDNA	2	57

* Total activity administered in 10-day period = 8.05 x 10^6 counts/min. Carbon-14 activity excreted in urine in 10-day period = 3.31 x 10^6 counts/min.

Results. Table I summarizes the distribution data for carbon-14 activity in the acid-soluble nucleotide and nucleic acid fractions of both kidney and liver tissue obtained from rats subjected to an experiment in which aminonucleoside was administered for a period of 10 days. In agreement with the earlier experiments(1), the acid-soluble nucleotide fraction obtained from kidney tissue was much more highly labeled than the comparable fraction



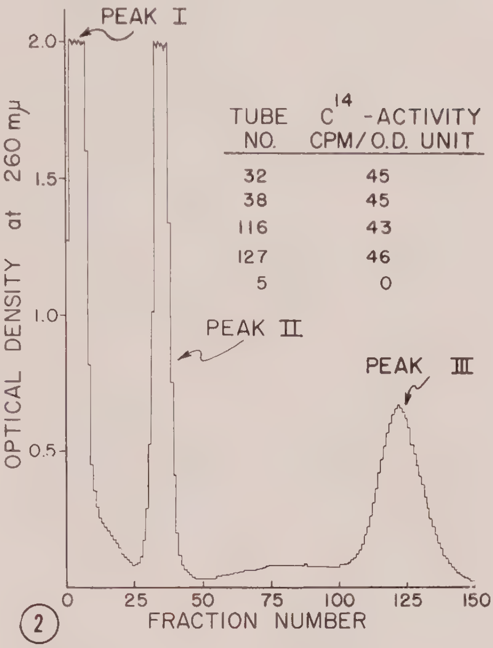


FIG. 1. Chromatography of acid-soluble nucleotide fraction of kidney. Chromatography on a 20×1 cm Dowex 1-X10 (formate) column of the acid-extract of 9.9 g pooled kidney tissue obtained from 6 rats, each of which had been injected subcutaneously with 10 mg aminonucleoside-8-C^{14} (2.3 μcuries) 3 hrs. prior to sacrifice. A total of 464 five ml fractions were collected. Eleven peaks, indicated by Roman numerals I-XI, were separated. Tubes, indicated by the bracket enclosures for each of the peak fractions, were pooled and concentrated by lyophilization. Optical densities recorded for tube Nos. 10, 11, 12, and 13 in Peak I were calculated from measurements made on suitable dilutions.

FIG. 2. Chromatography of acid-hydrolysate of liver BaRNA. Chromatography of acid-hydrolysate of 50 mg BaRNA on a 9×1 cm column Dowex 50-X12 cation exchange resin, developed with 2 N HCl at a flow rate of 0.8 ml/min. A total of 500 three ml fractions were collected. Pyrimidine nucleotides were eluted in Fractions 1-20. The purine base, guanine, was eluted in Fractions 28-42, and adenine in Fractions 105-140.

obtained from liver tissue. Results expressed in terms of counts/min./g tissue extracted, indicate 2.7 times as much carbon-14 activity in the acid-soluble nucleotide fraction of kidney tissue as was found in liver tissue. In contrast to this finding, however, are the specific activity data for kidney and liver RNA and DNA. In terms of counts/min./mg barium salt, liver RNA was 2.8 times as active as kidney RNA, and liver DNA 28 times as active as kidney DNA.

Detailed study of the chemical nature of carbon-14 activity in acid-soluble nucleotide fractions of both kidney and liver tissue revealed no difference between fractions obtained from tissues of rats sacrificed in as short a period as 3 hours after administration of a single large dose of aminonucleoside-8-C^{14} and similar fractions from rats given small daily injections of the material for periods as long as 10 days. Carbon-14 activity was found only in the nucleotides containing the purine bases adenine and guanine. Fig. 1 presents a typical chromatogram of the acid-soluble nucleotide fraction of pooled kidney tissue from 6 rats subjected to the acute experiment. Collection of 464 five ml fractions from the column revealed the presence of eleven 260 mμ-absorbing peaks. These were eluted from the column in the first 245 tubes. Carbon-14 activity determined on the peak tube from each of the 11 fractions indicated the presence of only 6 fractions labeled with the isotope. For purposes of comparison, the radioactivity of each of the peak tubes labeled with carbon-14 is presented in terms of total counts/min./optical density unit. Rechromatography of lyophilized Fractions I through VI on Dowex 1-X10 columns developed with the modified ammonium formate system(7), followed by identification of carbon-14 containing peaks, as described under methods, revealed the presence of only adenosine and guanosine phosphates. With the exception of Fractions II and III, which upon rechromatography gave only one peak, all other fractions were resolved into 2 or more peaks. No evidence was obtained for conversion of either aminonucleoside or its 6-monomethylaminopurine derivitive into the corresponding nucleotide.

Fig. 2 presents a typical chromatogram of the acid-hydrolysate of 50 mg of barium RNA isolated from livers of rats treated for a period of 10 days with aminonucleoside. Carbon-14 activity was found only in peaks II and III. Table II summarizes the spectral characterization data. For purposes of comparison, similar spectral data are included for adenine, guanine, 6-dimethylaminopurine and 6-methylaminopurine. Considered in conjunction with positions of elution of known pyrimidine nucleotides and the purine bases(6,9), it was concluded that peak II was guanine and peak III adenine. No evidence was obtained for the presence of carbon-14 labeled 6-methylamino- or 6-dimethylamino-purine in the acid-hydrolysates of either kidney or liver RNA or DNA.

TABLE II. Spectral Characterization Data for Purine Bases in HCl.

Base	Wave length		Absorbance ratios	
	Max.	Min.	250/260	280/260
	mμ	mμ		
Peak II	248	222	1.33	.64
" III	262	230	.93	.46
Adenine	264	228	.76	.37
Guanine	245	225	1.37	.74
6-Methylaminopurine*	267	232	.59	.75
6-Dimethylaminopurine	275	242	.82	1.07

* Values obtained from ultraviolet absorption spectrum given by Adler *et al* (9).

Discussion. Clearly these results and those previously reported(1) fail to provide evidence for the conversion of aminonucleoside to the corresponding aminonucleotide in the rat or for biosynthesis of nucleotides or other cofactors in which intact aminonucleoside, the 6-dimethylaminopurine moiety, or the 3-amino-3-deoxyribose moiety are substituted for normally occurring adenosine, 6-aminopurine, or ribose moieties. This would seem conclusively to eliminate from consideration a mechanism of action of aminonucleoside involving formation of abnormal or counterfeit nucleotides or cofactors which subsequently foul or inhibit metabolic pathways requiring the normally occurring adenosine compounds.

In the present studies no carbon-14 activity was found in either the pyrimidine nucleotides chromatographed from the acid-soluble nucleotide fractions of both kidney and liver tissue of aminonucleoside-nephrotic rats or in pyrimidine bases chromatographed from acid-hydrolysates of RNA and DNA isolated from the same tissues. Clearly labeling of cytidine nucleotide observed in metabolic studies with tritiated aminonucleoside(1) must have arisen as a result of cleavage of aminonucleoside at the glycosidic linkage and utilization of labeled ribose arising from the 3-amino-3-deoxyribose moiety in the biosynthesis of the cytidine nucleotide.

The observation of carbon-14 activity in adenine and guanine chromatographed from the acid hydrolysates of RNA and DNA clearly indicates metabolism of the 6-dimethylaminopurine moiety of aminonucleoside to precursor purine. Demethylation of the 6-dimethylamino group, accomplished either prior or subsequent to the splitting of the glycosidic linkage, would of course yield adenine, which could then be metabolized to guanine.

Although both the 6-methylamino- and the 6-dimethylamino-purine have reportedly been found in trace amounts in rat liver microsomal RNA(2), we were unable to detect, even with the assistance of the carbon-14 labeled purine moiety, the presence of either of the methylated bases in kidney or liver RNA or DNA isolated from the tissue residues remaining after extraction of the acid-soluble nucleotides with perchloric acid. While this observation might be explained in terms of differences in the purine base composition of microsomal RNA and that of RNA and DNA isolated as described, lack of evidence for the conversion of aminonucleoside to the corresponding aminonucleotide would seem to preclude incorporation of either of the labeled 6-methylamino- or 6-dimethylamino-purine moieties into nucleic acids.

Summary. Detailed studies were made of the chemical nature of carbon-14 activity found in acid-soluble nucleotide, ribonucleic acid, and deoxyribonucleic acid fractions isolated from kidney or liver tissue of rats rendered experimentally nephrotic with a series of 10 small daily injections of aminonucleoside-8-C^{14}, and, in similar fractions obtained from rats sacrificed as early as 3 hours after administration of a single large dose of the labeled compound. No significant differences were noted. Only the adenosine and guanosine phosphates of the acid-soluble nucleotide fractions and the adenine and guanine bases of RNA and DNA hydrolysates were labeled with carbon-14. No evidence was obtained either for conversion of the intact aminonucleoside to the corresponding nucleotide or for incorporation of the 6-dimethylamino- or 6-methylamino-purine moieties into ribonucleic acid or deoxyribonucleic acid of kidney or liver tissue.

The author wishes to acknowledge the technical assistance of Mr. Leophas Ford.

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Effect of Adrenal Tissue on Desoxycorticosterone Hypertension.* (27012)

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Swingle and coworkers, comparing 7 adrenalectomized and 4 intact dogs, noted that a given amount of desoxycorticosterone produced a lesser degree of hypertension in the intact dogs(1). Soffer also found it difficult to produce hpyertension with desoxycorticosterone in patients with intact adrenals, even though it occurred readily in patients with adrenal insufficiency(2). In this study we wanted to find out whether the presence or absence of adrenal tissue has any effect on development of desoxycorticosterone hypertension in the rat. We carried out our study on 2 groups of rats, producing hypertension by administering a given amount of desoxycorticosterone to both groups. In one group both adrenals had been removed; in the other group, both adrenals were intact.

Methods. Forty male Wistar rats, of approximately 100 g, were each injected subcutaneously with 31 mg of desoxycorticosterone trimethylacetate. Four weeks later the same dose was repeated. Half of these rats were given bilateral adrenalectomies; the other half received sham operations with adrenal tissue left intact. All 40 were allowed to drink only .5% saline and were fed only Purina lab chow.

Between the sixth and seventh week after original injection, 3 blood pressure readings, at 2-day intervals, were taken on each rat, using the microphonic method(3). The average of these was considered the "true" blood pressure.

Results. Blood pressures were obtained on 18 rats in the adrenalectomized group and on 15 in the group receiving sham operations. The mean blood pressure of the adrenalectomized group was found to be 174 mm Hg. (S.D. ±9); that of the group whose adrenals were left intact was 178 mm Hg. (S.D. ±19). The

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difference between the 2 groups was not statistically significant ($p = .5$). Both groups were quite hypertensive, since normal male Wistar rats of the same age have an average blood pressure of approximately 105 mm Hg.

Conclusions. These results indicate that in rats under these conditions, presence or absence of adrenal tissue has no effect on the severity of hypertension produced by desoxycorticosterone administration.

The authors would like to acknowledge gratefully the gift of desoxycorticosterone from Dr. Robert Gaunt, Ciba Corp.

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Hemadsorption in Monkey Kidney Cell Cultures of Mycoplasma (PPLO) Recovered from Rats.* (27013)

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(Introduced by John P. Fox)
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The hemadsorption test for viral agents in cell cultures has been widely utilized(1). This report presents evidence that a PPLO isolated from the respiratory tract of laboratory rats causes hemadsorption and cytopathic changes (CPE) in monkey kidney cell (MKC) cultures. The isolations were made during etiological investigation of chronic respiratory disease (CRD) in these animals(2).

Materials and Methods. Preparation of rat specimens. Three 2-3 month old Sprague-Dawley rats exhibiting signs of CRD were furnished by Dr. Benjamin N. Berg(3). Gross examination revealed abnormal amounts of upper airway secretion and pulmonary consolidations. Lung and tracheal tissues were ground in diluting fluid consisting of Hanks' balanced salt solution (BSS) containing 0.5% lactalbumin hydrolysate (LAH), penicillin, streptomycin and mycostatin. Supernatant fluids were collected after centrifugation and inoculated directly into cultures or stored at −60°C. Airway washings were inoculated immediately.

Cell cultures. Primary rhesus MKC cultures were initially nourished with BSS containing 0.5% LAH and 2-10% calf serum. Prior to use, cultures were refed with Eagle's basal medium (4) prepared with BSS. Penicillin, steptomycin and mycostatin were added to both media. Incubation was at 36-37°C.

Assay and detection in MKC cultures. Serial 10-fold dilutions of material to be tested were prepared in diluting fluid. Four cultures were inoculated with 0.1 ml of each dilution. Cultures were observed for CPE and were tested usually after 4 to 5 days for hemadsorption of guinea pig erythocytes according to the technic of Vogel and Shelokov(1) as modified by Chanock *et al*(5). Titers were calculated by the Reed-Muench method and expressed as the log $TCD_{50}/0.1$ ml of inoculum.

PPLO broth and agar cultures. Test material was inoculated into Bacto-PPLO broth(6). After 2-4 days aliquots were sub-inoculated in broth or spread over the surface of Bacto-PPLO agar(6). Both broth and agar contained 3% PPLO serum fraction(7), penicillin and streptomycin. Incubation was at 36-37°C. Agar plates were observed microscopically for typical PPLO colonies, recognition of which was periodically verified by the stained agar technic of Dienes(8). No bacterial growth

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was observed on several occasions when fluids known to contain PPLO were inoculated into thioglycollate broth.

Virus. Sendai virus strain D/Sendai/52 was obtained from Dr. Keith Jensen. Material from a MKC culture passage was used.

PPLO. A strain (HPPLO) isolated from HeLa cell cultures and passed 205 times in broth and a strain (MPPLO) isolated from mouse lung and passed 4 times in broth were furnished by Dr. John B. Nelson. The strains isolated from rats in this work will be referred to as rat PPLO (RPPLO).

Experimental. Three changes occurred in MKC cultures inoculated with respiratory tract material from each of the 3 rats. First, after one week CPE was observed which was characterized by sharply demarcated holes bordered by pyknotic and fragmented cells (Fig. 1c). Examination after staining with hematoxylin and eosin revealed numerous tiny eosinophilic, cytoplasmic granules similar to those previously noted in PPLO infected cell cultures(9). Ultimately the entire sheet was destroyed. Second, coincident with CPE, culture fluids while remaining clear became distinctly more acidic than in controls. Third, hemadsorption tests were positive 9 days after inoculation (Fig 1a). In subsequent subcultures these changes appeared within 4-5 days. It was shown in the following experiment that RPPLO can induce these effects.

A specimen from a single rat was inoculated into MKC and PPLO broth cultures. In the second broth transfer RPPLO was demonstrated on agar from which colonies were removed to initiate 10 more broth passages. Material from the final passage was RPPLO positive on agar and in MKC cultures exhibited characteristic CPE. The log infectivity titer of this material determined by hemadsorption was 6.5 per 0.1 ml. In cell cultures the transmissible effects mentioned above were evident. After 3 subcultures the log infectivity titer of the fluid determined by hemadsorption was 6.0 per 0.1 ml and RPPLO was recovered on agar from this final passage material. Fluids from uninoculated MKC cultures were passed in parallel and hemadsorption was never observed nor was RPPLO recovered.

Characteristically RPPLO continued to cause hemadsorption after repeated passage in MKC cultures followed by serial transfer in broth. In an isolated case, after 8 cell and 18 broth culture passages hemadsorption became irregular.

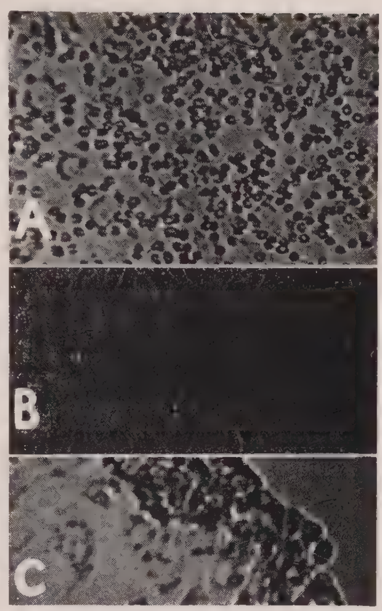

FIG. 1. a) Positive hemadsorption test in monkey kidney cell culture 4 days after inoculation with RPPLO. b) Similarly treated uninoculated culture. c) Typical RPPLO CPE prior to addition of erythrocytes (×140).

The following experiments demonstrated that inactivated RPPLO failed to induce the observed changes in cell culture. Broth and cell culture fluids containing 10^3-10^5 $TCD_{50}/0.1$ ml of RPPLO were either heated for 15 minutes at 50°C, rapidly frozen and thawed 5x or exposed to kanamycin sulfate (125 μg/ml) for 48 hours at 4°C. After each treatment RPPLO could not be recovered on agar nor were CPE or hemadsorption observed following inoculation of MKC cultures. Furthermore, after 5 days of incubation fluids from these cultures were negative for RPPLO when tested on agar. Similar exposure of Sendai virus to kanamycin failed to inhibit the usual development of CPE and hemadsorption in MKC cultures, indicating that kanamycin itself did not prevent the occurrence of hemadsorption in such cultures.

Certain features of the hemadsorption test were studied. Hemadsorption became maximum 4 days following incubation and was strongest 20-30 minutes after incubation with erythrocytes at either 6°C or 36°C. Shaking detached red cells more easily than in the case of cultures infected with Sendai virus. That hemadsorption is an accurate index of the presence of RPPLO in MKC cultures was shown in one titration when organisms were recovered only from cultures positive for hemadsorption. Rarely heavily inoculated cultures failed to hemadsorb, or did so weakly, while diluted material yielded positive tests.

HPPLO and MPPLO were the only other strains tested for hemadsorption in MKC cultures. The latter was cytopathogenic; however, under the experimental conditions employed neither strain caused hemadsorption.

Discussion. Chronic respiratory disease is endemic in most laboratory rat colonies(3), and is reportedly comprised of 2 pathological entities; namely, infectious catarrh and enzootic bronchiectasis(2). Infectious catarrh has been associated with PPLO(10), whereas there is evidence for a viral etiology of enzootic bronchiectasis(11). Investigation of CRD is complicated by the apparent frequent clinical and pathological overlapping of the two conditions. During attempts to isolate the virus in MKC cultures hemadsorption was observed. This effect was caused by RPPLO which was consistently isolated from inoculated, hemadsorption positive cultures. PPLO was never recovered from control culture fluids.

Evidence that hemadsorption was not due to a latent simian agent(12) is as follows: During the course of each experiment repeated testing of uninoculated MKC cultures prepared from the same monkey revealed no hemadsorption. When fluids from uninoculated cell cultures were passed serially, in parallel with experimental fluids, hemadsorption tests were consistently negative. In addition, fluids from experimental tubes failed to hemagglutinate chick erythrocytes, a property shared by some noncytopathogenic simian agents(12).

Dependency of the observed changes on the presence of an accompanying virus of rat origin was excluded by the demonstration that hemadsorption and CPE were associated with RPPLO following its isolation and serial passage in broth and agar cultures. The organism continued to hemadsorb after as many as 14 cell free subcultures.

Attention is drawn to behavioral similarities shared *in vitro* by certain viruses and PPLO. Both microorganisms may multiply and cause cytopathic effect in cell cultures(13). Both may agglutinate erythrocytes(14), and the present paper describes the ability of a PPLO to cause hemadsorption, a property shared by many viruses.

The ability to cause hemadsorption may prove useful in classifying strains of mycoplasma.

Summary. Hemadsorption of guinea pig erythrocytes by MKC cultures infected with a PPLO from the respiratory tract of laboratory rats is reported.

We express our gratitude to Dr. Benjamin Berg and Dr. John Nelson for their stimulation and assistance.

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Histochemical Demonstration of Mammalian Glucosidase By Means of 3-(5-Bromoindolyl)-β-D-glucopyranoside.* (27014)

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Because of the small amount of glucosidase in mammalian tissue, there is a need for a sensitive substrate for its demonstration in tissue sections. The bromo substituted indole derivitives seemed appropriate because of their relative ease of enzymic hydrolysis as well as the nature of the final reaction product which is highly colored, particulate and insoluble. The 3-(5-Bromoindolyl)-β-D-glucopyranoside was, therefore, synthesized in our laboratory by Dr. Jerome Horwitz and his associates for this purpose in 1958. When this was tested histochemically on mammalian tissue sections, it provided promising results.

This report describes the distribution and localization in tissue of this enzyme and indicates improved synthesis of this substrate.

The principle of the histochemical reaction is similar to that of 5-bromoindoxyl acetate (1,2,3) and is illustrated in Fig.5. The glucosidase in tissue causes hydrolysis of the colorless 3-(5-Bromoindolyl)-β-D-glucopyranoside (I) to form an intermediate, 5-bromoindoxyl (II) which is unstable and is readily transformed to the highly insoluble intensely chromogenic 5,5′-dibromoindigo (III). This is precipitated and can be seen readily at sites of enzymic activity in tissues and cells. The results are quite distinctive and different from those obtained in the study of esterases(1,2,3).

Materials and Methods. The 3-(5-Bromoindolyl)-β-D-glucopyranoside was prepared by Dr. Horwitz and his associates in the organic laboratory of the Detroit Inst. of Cancer Research. The preparation was as follows:

To a cold (−5°C) solution of methanol (100 ml) containing 1.4 g (0.058 g atom) of sodium was added 15 g (0.059 mole) of 5-bromoindoxyl acetate and the greenish brown solution stirred magnetically for 1 hour under an atmosphere of nitrogen. The reaction mixture was then cooled to 0°C and a solution of 24 g (0.059 mole) of acetobromoglucose in 100 ml of methanol was added all at once. The mixture was stirred for 18 hours during which time the reaction mixture was allowed to reach room temperature. The solvent was then evaporated under diminished pressure; water was added to the residue and a stream of air passed into the mixture for *ca* 0.25 hour. The insoluble material was removed by filtration and the filter cake then triturated with 30 ml of warm N,N-dimethylformamide. Norit was added to the extract and the mixture filtered through Celite. The clear filtrate was evaporated to dryness under reduced pressure; the brown residue was suspended in water and filtered: wt. 6.6 g (30% yield), m.p. 245-247° dec. The product was recrystallized from aqueous pyridine to give a colorless solid m.p. 260-261° dec., ∝D 25°-59° (C=1, 50% aqueous dimethylformamide.)

Analysis:

Calculated for $C_{14}H_{16}BrNO_6$:
$C=44.93 \quad H=4.31 \quad N=3.74$
Found: $C=45.07 \quad H=4.32 \quad N=3.83$

The same glucoside was initially obtained from 5-bromoindoxylic acid by a procedure similar to that described by Robertson(4) for synthesis of 3-indolyl-β-D-glucopyranoside. However, the method is tedious and overall yield is poor. Accordingly, the procedure described above is the method of choice. Recently a different procedure for preparation of (I) was reported(5).

Rats, guinea pigs and mice were used for this study. The tissues were cut into blocks 2-4 mm thick, placed on small pieces of filter paper, inserted into glass tubes and immersed in Dewar flasks containing acetone and dry ice at a temperature of about −70°C. The frozen blocks were transferred to a Linderstrom-Lang cryostat and cut at 6μ at −20°C.

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Tissues were affixed to slides and stored at −25°C until ready for use. These slides were placed in a staining rack and immersed immediately into a precooled pretreatment solution (−65°C) consisting of n-butanol, acetone and chloroform (ratio 1:1:1 by volume) in order to remove lipids(6). After remaining in the solvent mixture for 30 minutes the staining rack containing the slides was removed and immediately washed in water with agitation. Three rinses were sufficient to remove the solvent from the tissues. The slides were placed in Coplin jars containing an incubating solution for 3 hours at room temperature (27°C). The composition of the incubating solution was as follows:

4 mg 3-(5-Bromoindolyl)-β-D-glucopyranoside in 1 ml of dimethylformamide.
5 mg PVP in 1 ml of water.
14 ml acetate buffer, 0.2M, pH 5.4.

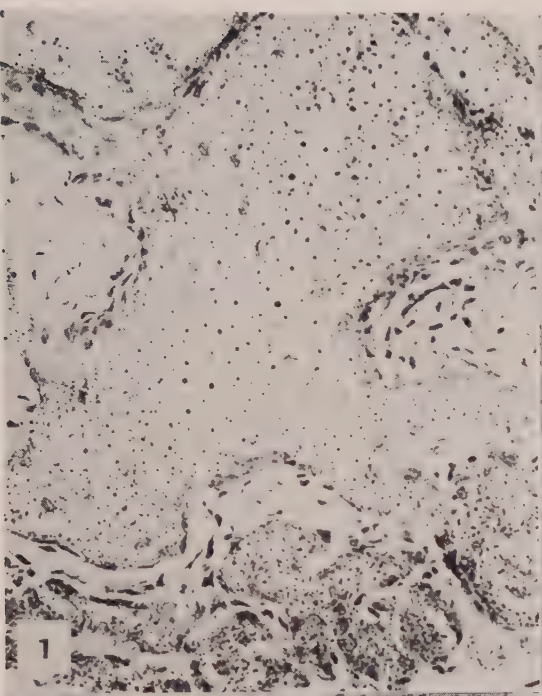
After incubation the slides were removed from the Coplin jars and rinsed in water and fixed in 40% formaldehyde for 1 minute followed by a thorough rinsing in water. The slides were then stained with Mayer's Haem Alum for 6 minutes, decolorized by 1% aqueous acetic acid and counterstained with a saturated aqueous solution of picric acid for 4 minutes. They were then rapidly passed through a series of graded alcohols to xylene and mounted in balsam. Slides not counterstained were mounted directly in glycerogel or run through alcohols to xylene and mounted in balsam.

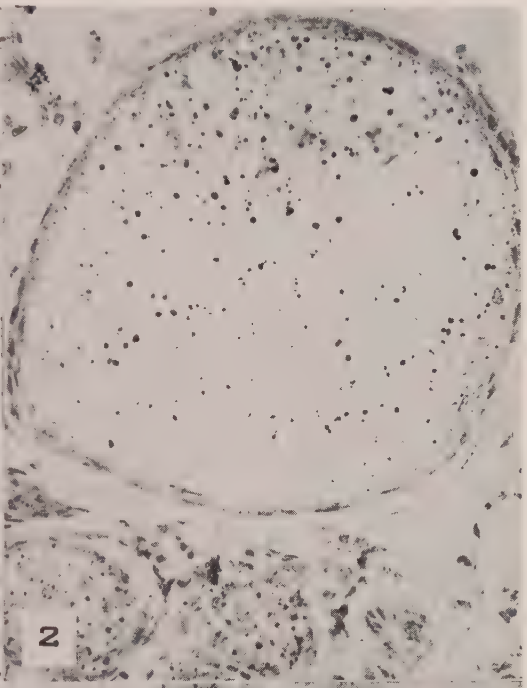
Results. In general the enzyme activity was greatest in the preputial glands, duodenum and small intestine with traces in the adrenals. liver, kidney, parotid, pancreas, stomach, spleen, lymph node, epididymis and skin. No reaction was present in the thymus, lung, cerebrum, cerebellum, heart, skeletal muscle, tongue, esophagus, uterus, ovary, oviduct, testes and prostate. This was fairly constant in all species examined with minor variations. Only the outstanding features will be described.

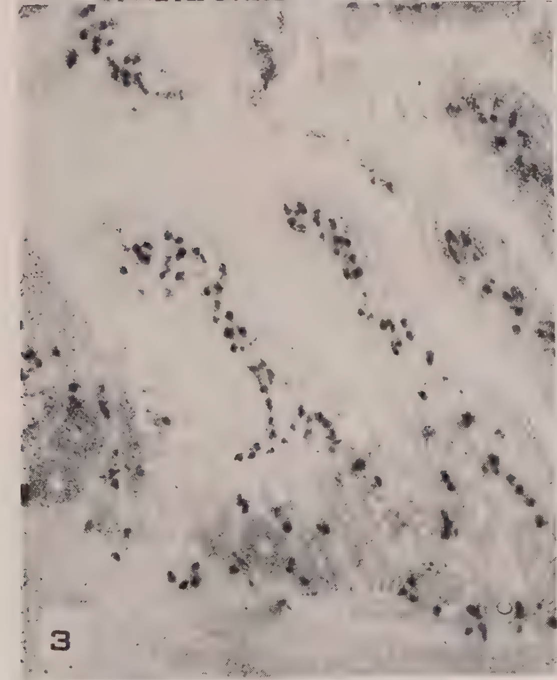
In the preputial gland all the cells of the acinar tissues showed marked enzyme activity, although this varied depending upon age and degeneration of the acini. In small young primordial acini, where the cells were uniform and showed no signs of central degeneration, fine blue granules (III) were dispersed throughout the cytoplasm. In the more mature acini the reaction was more intense and resultant granules larger. In the ductal system, which cannot be conveniently separated from the acini because of their intimate association, degree of intensity was greater with larger granules. The surrounding stratified epithelium was negative (Fig. 1). Areas within the ductal system where definite sebum was present showed an intense activity with accumulation of blue dense granules lying free within the secretory products of the gland (Fig. 2). The stroma of the gland was negative.

The duodenum and small intestine revealed moderate reaction. The large reticulum cells within the villous stalks showed a fairly comparable reaction to that seen in the preputial gland. Only the cytoplasm was positive. The granules were small (Fig. 3,4). A very slight reaction was present in the cells of the intestinal gland. This was different from the esterase reaction with 5-bromoindoxyl acetate which showed intense reaction in these cells but none in the villous stalks. Similar reaction was present in the reticulum cells of the stomach and large intestine, but these cells were extremely sparse.

The adrenals gave a consistently uniform reaction in the cytoplasm of all the cells of the cortex. The granules are larger than those of the liver and kidney and there was an accentuation of these in the glomerular layer. Most of the granules were situated at the peripheral border of the cell. The liver and kidney gave a trace reaction with very fine somewhat elongated granules. Only the cytoplasm was involved. Occasionally the Kupffer cells showed a marked activity. In the kidney only the cytoplasm of the proximal convoluted tubules was involved, but the granules were sparse in comparison with those of the liver. The pancreas revealed fine elongated granules in the cytoplasm of the parenchymatous, but not the insular cells. In the parotid and to some extent the submaxillary gland only the larger ducts showed granules in the cytoplasm. In the spleen only certain reticulum cells in the peripheral sinusoids had granules in their cytoplasm. Not all reticulum cells gave the reaction. Those that did varied in intensity in different animals. A slight diffuse reaction







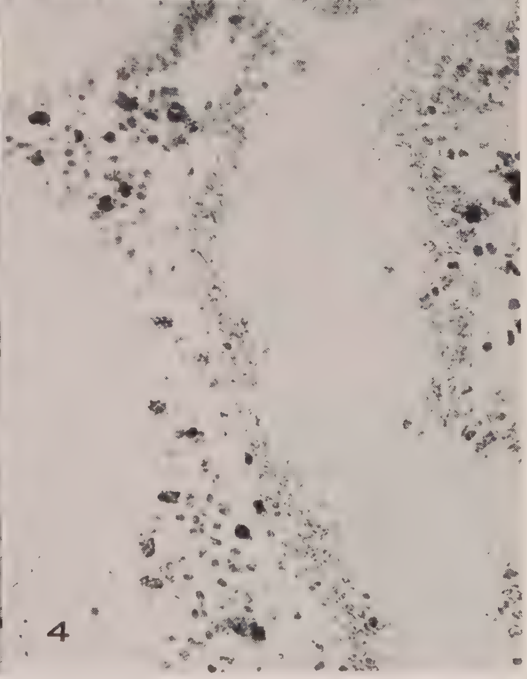


FIG. 1. Preputial gland showing β-glucosidase reaction in acinar cells with varying size granules. Frozen section. Counterstained. X300.

FIG. 2. Preputial gland showing β-glucosidase. Small acini lower left shows discrete enzyme activity in cells. Duct with degeneration showing enzyme activity in sebum. Frozen section. Counterstained. X300.

FIG. 3. Small intestine showing β-glucosidase activity in reticulum cells of villous stalks. Slight activity in mucosal epithelium. Frozen section. Not counterstained. X120.

FIG. 4. Small intestine showing β-glucosidase activity in reticulum cells of villous stalks. Higher power to show granular size in cell cytoplasm. Frozen section. Counterstained. X300.

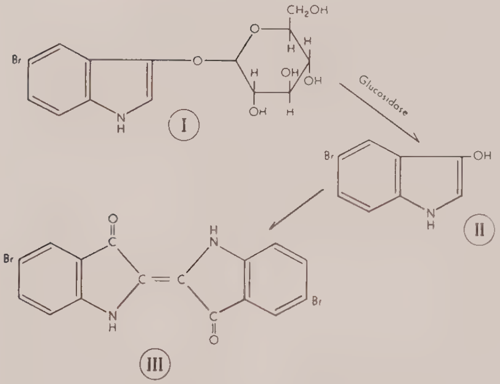


FIG. 5. Substrate Reaction for Tissue Glucosidase.

was present throughout the spleen. The lymph node gave a positive reaction in a few reticulum cells; however, they were not as numerous as those of the spleen. Reticulum cells in the stroma of the epididymis gave a good reaction but they were few in number. The sebaceous glands of the skin gave a good reaction in the cytoplasm and appeared as fairly large, rounded, sparse granules. As a rule, there were only a few granules per cell, but these were consistent.

Characterization of Tissue Glucosidase. When slides with the tissue were placed in a water bath (58°C) for 1 hour and then incubated in the substrate solution, no hydrolysis of the substrate (I) occurred, indicating that the enzyme was destroyed by this process. When the potassium salt of ferri-ferrocyanide (0.05M) was added to the substrate solution and the slides incubated no hydrolysis took place. When 2% formalin was placed in the incubating media for 3 hours, no hydrolysis occurred and the final reaction was negative. The enzyme is sensitive to formalin. When copper sulphate was added to the substrate solution (1 ml of 0.03M) and the slides incubated, no reaction occurred.

Boiled saccharate was used to produce saccharo-1-4-lactone which is a strong inhibitor of glucuronidase(7). This was made up fresh, cooled and added to the incubating solution (1 ml of 0.05M). A series of slides incubated in this solution showed no inhibition of hydrolysis (I) and the final reaction product was the same as in the slides incubated without saccharate.

A high concentration of the salt (I) caused inhibition of the reaction, so a series of 9 substrate solutions were set up containing graded final concentrations of (I) from 0.006 M to 0.00007M. Optimal concentration was found to be 0.0006M. This inhibitory effect has been noted for intestinal β-glucosidase using phenol substrates(8).

To determine an optimal pH, a series of 8 substrates were prepared with pH's ranging from 2.0 to 8.8. No reaction occurred below a pH of 5.0 or above 6.2. Optimum pH was 5.4.

Discussion. Although β-glucosidase is an enzyme which occurs mainly in plants and fungi, small amounts occur in mammalian tissue. Recently, 2 investigators(9,10) have made surveys of its occurrence in mammalian tissue using homogenate methods. The substrates used were 6-bromo-2-naphthyl-β-D-glucopyranoside and 4-methylumbelliferone-β-glucoside, respectively. In the former(9) the glucosidase splits off the glycone leaving the aglycone which is coupled with tetrazotized diorthoanisidine under alkaline conditions. The colored final product was measured colorimetrically. Attempts were made to use this method histochemically on tissue sections, but an entirely satisfactory result was not achieved. The latter(10) employed an extremely sensitive method using 4-methylumbelliferone-β-glucoside. This was prepared by the authors fresh daily as it decomposed readily. The glucosidase in the tissue split off the glucose leaving the aglycone which was measured fluorometrically.

In both of these studies the intestine had a high activity. Liver and kidney also showed activity. In the former study the guinea pig pancreas was very active(9). In our histochemical investigations we did not find this disproportion. The preputial gland was not studied in either series. It should be appreciated that extremely small amounts in various tissues were demonstrated by these studies.

In our studies definite enzyme activity was demonstrated in the preputial gland, small intestine and the adrenal gland. In other organs such as the liver and kidney only trace amounts were demonstrated. In the reticulo-endothelial cells of the sinus reticulum of the spleen and lymph node good reactions were present. This, however, was variable as not all of the reticulum cells showed the same intensity. Reticulum cells of the thymus were negative. Under certain conditions the Kupffer cells of the liver showed a strong reaction. The conditions responsible for this variation are being studied.

Summary. The 3-(5-Bromoindolyl)-β-D-glucopyranoside (I) was prepared in our laboratory for histochemical study of tissue β-glucosidase. The final reaction product following incubation of tissue sections was 5,5′-dibromoindigo which is highly chromogenic and precipitates in cells and tissue at the site of enzymic activity. The enzyme is readily destroyed in tissue by ferri-ferrocyanide and formalin, which is not the case with nonspecific esterase or glucuronidase. Saccharo-1-4-lactone, which has an inhibitory effect on β-glucuronidase, does not affect β-glucosidase. "Substrate inhibition" can also be demonstrated. Optimum pH is 5.4. The β-glucosidase can be demonstrated best in the preputial gland where activity is high. The older acini of the gland showed more intense reaction. Reticulum cells within the villous stalks of the small intestine showed a good reaction. Reticulum cells of the spleen, lymph node and Kupffer cells showed a varied reaction. Extremely small amounts are present in the liver and kidney.

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Diagnosis of Human Allergy Utilizing Passive Skin-Sensitization in the Monkey *Macaca irus.* (27015)

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If blood serum from an actively allergic individual is injected into the skin of a nonallergic volunteer the skin of the recipient may become sensitized in the immediate area of the serum injection. This phenomenon is referred to as passive sensitization. The passively sensitized skin site will often exhibit an inflammatory reaction with wheal formation when injected with a minute amount of the substance(s) causing allergy in the serum donor. This particular direct local application of the phenomenon of passive cutaneous anaphylaxis (P.C.A.) in humans is referred to as the "Prausnitz-Küstner test" or the "P.-K. test" (1). The P.-K. test has been used extensively as a diagnostic aid in identifying the substances responsible for allergies, and is especially useful in allergies of infants, debilitated children and severely allergic older patients.

The P.-K. test presents the grave danger of transfer of serum hepatitis infection to the human volunteers. Because of this danger of infection we avoided the use of the technique in testing the sera of those factory employees showing signs of sensitivity to castorbean dust.

Ovary(2) has discussed the phenomenon of passive cutaneous anaphylaxis in guinea pigs and other lower animals. He suggested that a colloidal dye such as Evans Blue be injected intravenously along with the antigen, in order to make more visible the inflamed area resulting from antigen-antibody reaction. Dye leakage into the extravascular spaces (extravasation of dye) results from the increased permeability of the minute blood vessels in the inflamed areas. Layton and coworkers(3) have adapted this technique to the problem of detecting seed antigens of *Ricinus communis* (castorbean).

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Tests with rabbit antisera in the skins of guinea pigs are not of themselves adequate criteria of allergenicity to humans, hence any conclusions relating to allergenicity must ultimately rely upon human reactions to the allergen or the use of human material in testing. Because of this essential criterion and the hazards involved in passive sensitization of human volunteers, the possibility of using monkeys was investigated.

Grove(4) attempted to sensitize the skins of chimpanzees and lower monkeys with serum from allergic humans and concluded that passive transfer to these primates was not feasible. Nevertheless, the need to determine the allergenicity of isolated castorbean antigens(5,6, 7,) suggested attempts to produce passive sensitization by transfer of human serum reagins in another species of primate. The Philippine crab-eating monkey, *Macaca irus,* was a first choice because of its light skin, small size, gentle nature, relative abundance, and low price.

Preliminary tests conducted upon 4 female monkeys showed(8) that human reagins were bound in the monkey's skin but that not less than 24 hours nor more than 72 hours time should be allowed for attaining optimum reactivity in the sensitized sites. It was also found that extreme fear and physical struggle would inhibit wheal formation without affecting extravasation of intravenously injected Evans Blue dye. Light tranquilization with reserpine appeared to enhance reactivity in the monkey.

After demonstrating that human serum reagins would passively sensitize this species of monkey, an effort was made to standardize the techniques involved.

Experimental. Development of a Diagnostic Procedure. Sera were available from blood samples drawn 15 months earlier from employees who were exhibiting symptoms of allergy during a castorbean processing run. These patients had been referred to local general medical practitioners, but not to allergy specialists, and were diagnosed as probably allergic to castorbean meal. The blood serum samples were taken 4 days after the appearance of symptoms, and had been preserved by freezing and storage at −10°C.

A sample of partially purified, heat-stabile, water-soluble castorbean seed protein was available from a joint project with Stanford Research Institute and was coded CB-S.R.I.(3,6). This antigenic material was dissolved in physiological saline solution to give a solution containing approximately 10 mg of protein per ml. of solution.

Five male and 5 female monkeys of the species *Macaca irus* were purchased from a local animal importer. These animals weighed from 2 to 3 lbs and appeared to be free of infection and skin lesions.

Procedure: The hair was clipped from the abdomen, upper arms, thighs, cheeks, chin and eyebrows of each animal. The bare skin was wiped with 80% ethanol and while yet damp the abdomen was marked off with a red wax pencil into 10 areas, 5 on either side of the midline, to give a total of 19 ventral sites on each animal. With this arrangement 9 serum samples could be tested in duplicate against one antigen on the light ventral skin surfaces of each monkey.

Each site was injected intradermally with 0.05 to 0.1 ml. of undiluted human serum using a No. 27 needle and a 1-ml. tuberculin syringe. One or more sera from individuals known to be nonallergic were always included as negative controls. After the sensitizing injections the monkeys were returned to their cages, and 24 hours were allowed to elapse before conducting the local skin tests for sensitivity. The local skin tests were conducted in a manner similar to that described by Prausnitz and Küstner(1) for tests on passively sensitized humans; a minute drop of the selected saline solution of antigen was injected intradermally into the sensitized site at or near the the orginal needle mark. Only one antigen or antigenic fraction was tested in an individual monkey because of diffusion of the antigen from the site of injection thereby causing wheal formation in nearby sensitized sites.

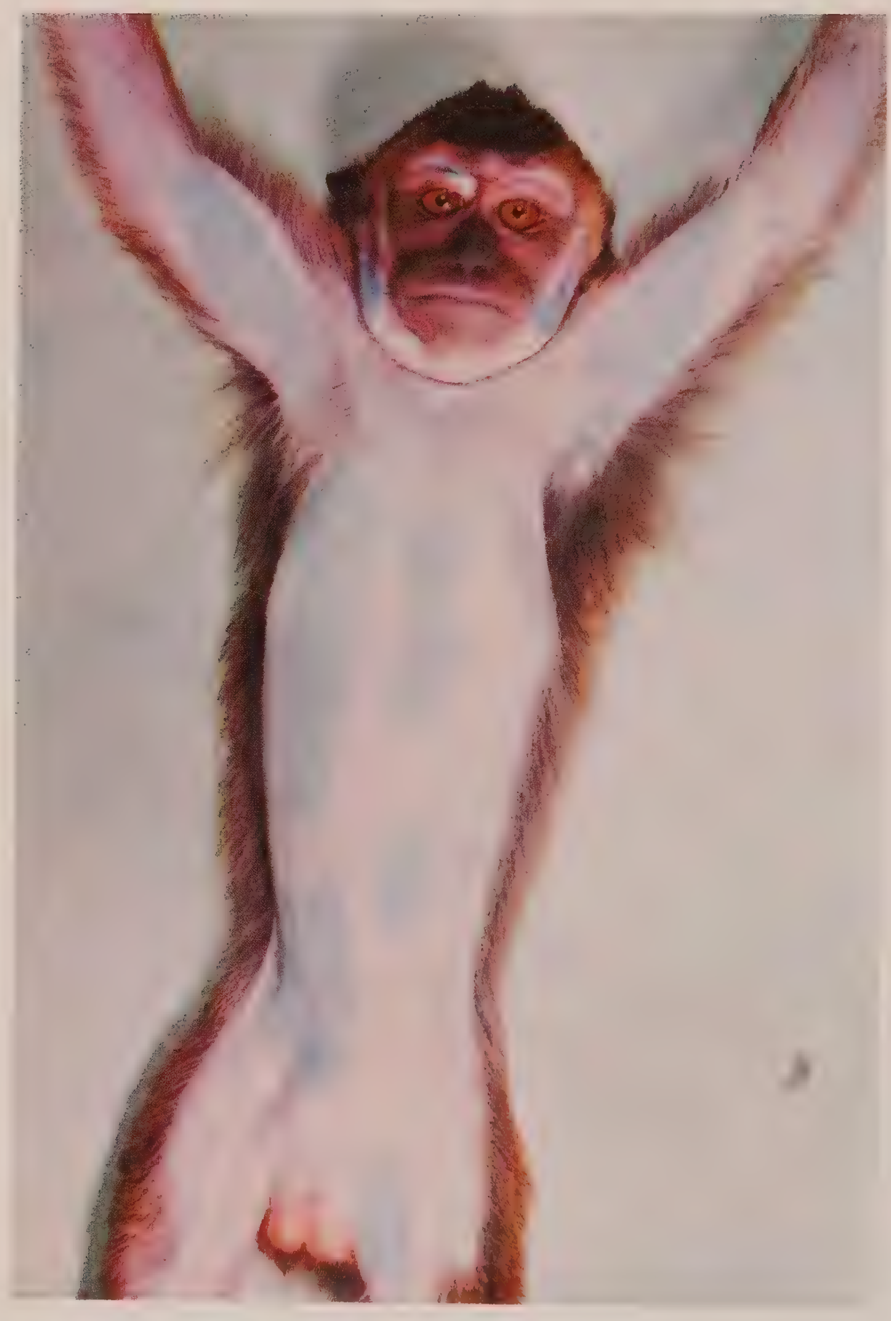

FIG. 1. Passive cutaneous anaphylaxis in *Macaca irus* sensitized with sera from allergic humans. Extravasation of Evans Blue in sensitive sites. No reaction in control sites (B.R.); see sketch in Fig. 2.

In certain very calm monkeys it was observed that wheal formation occurred in the orbital and cheek sites 10 minutes after injection of 1 mg of CB-S.R.I. intradermally into one of the abdominal sites. A similar but more immediate multiple-site reaction was obtained by injecting 0.1 mg of the antigenic material into the heart or preferably into a saphenous vein. Multiple-site reactions to an injection of the allergenic material were easily read and interpreted when the colloidal dye, Evans Blue, had been injected intravenously 5 to 10 minutes prior to challenge with the allergenic material. With monkeys weighing 2 to 3 lb 1.5 to 2.0 ml of 0.5% solution of Evans Blue in physiological saline was injected directly into a saphenous vein, surgically exposed under local anesthesia with adrenaline-free procaine which did not affect the P.C.A. reactions.

Results. A photograph of a multiple-site passive reaction to castorbean seed protein is shown in Fig. 1. The blue spots are the result of extravasation of the colloidal dye due to localized increase in vascular permeability induced by the castor allergen-antibody reaction (histamine release) in the sensitized sites. This phenomenon is comparable to the P.C.A. reaction(2) in the guinea pig passively sensitized with rabbit anti-castor serum(3). Fig. 2 is a sketch giving the code to Fig. 1. The same type of reaction without the dye is shown in the photograph (Fig. 3) of large wheals in sensitized sites (K. H. serum) on the eyelids of a monkey tested by intradermal injection of CB-S.R.I. into sites on the abdomen. Those abdominal sites which had been injected with sera from castorbean-allergic individuals exhibited the wheal formation characteristic of the corresponding Prausnitz-Küstner reaction in passively sensitized humans. The observations are tabulated in Table 1.

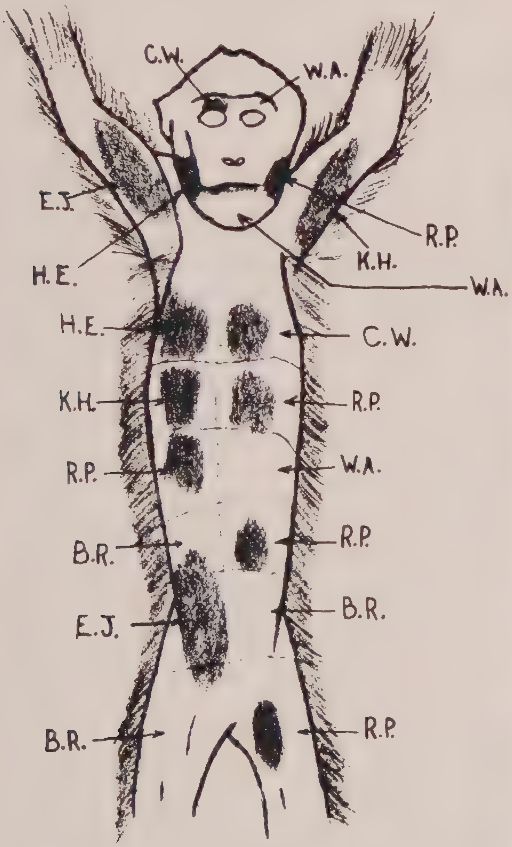

Fig. 2. Initials of donors identifying sources of human sera giving positive passive cutaneous anaphylaxis in *Macaca irus* challenged intravenously with castorbean antigens. See photograph, Fig. 1. (K. H. is not an employee, but is the wife of a nonallergic employee. She reacts to dust from his work clothing).

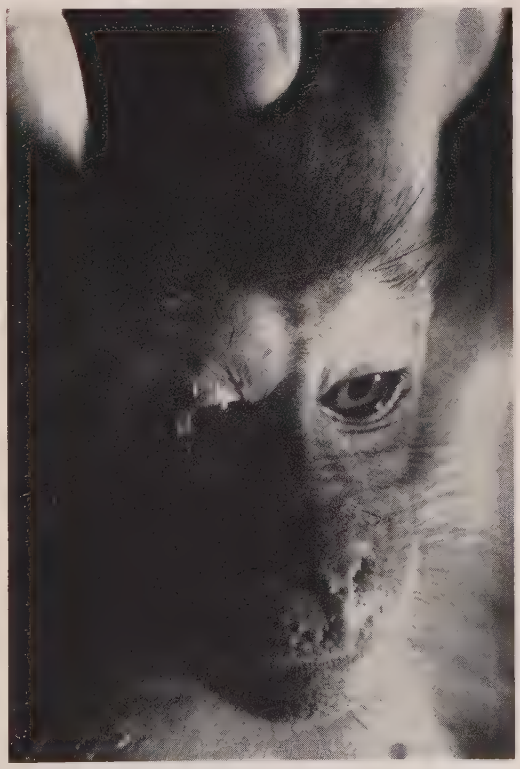

Fig. 3. Large wheals in sensitized sites on upper eyelids of *Macaca irus*.

TABLE I. Passive Skin Testing of Human Sera in *Macaca irus* for Allergy to *Ricinus communis* (Castorbean)

Human serum donor	Symptoms	Reaction in passively sensitized monkeys: Wheal formation in direct skin tests (6 monkeys)	Reaction in passively sensitized monkeys: Extravasation of Iv.* injected Evans Blue (4 monkeys)
1. W.A.	asthma**	negative 6/6	negative 4/4
2. A.C.	Henoch's purpura**	" 6/6	" 4/4
3. R.P.	asthma, rhinitis	positive 6/6	positive 4/4
4. W.R.	asthma, conjunctivitis	" 6/6	" 4/4
5. K.H.	acute asthma	" 6/6	" 4/4
6. M.H.	asthma	" 6/6	" 4/4
7. C.W.	"	" 6/6	" 4/4
8. H.E.	"	" 6/6	" 4/4
9. C.H.	hay fever, asthma	" 4/4	" 2/2
10. B.R.	none (control)***	negative 6/6	negative 4/4
11. E.J.	hay fever, asthma	positive 6/6	positive 4/4
12. H.S.	burning nose	negative 4/4	negative 2/2
13. E.F.	none (control)***	" 4/4	" 2/2
14. J.S.	" " ***	" 4/4	" 2/2

* Intravenous injection.

** These employees may be sensitive to some castor constituent not present in CB-S.R.I.

*** Sera collected Nov. 1960; all other sera were collected Dec. 1959-Jan. 1960.

After the studies on monkeys, we made a screening study by skin tests of 107 personnel of a castorbean processing factory (in cooperation with a licensed physician) and confirmed the results of the monkey tests. The results of the clinical tests will be presented later.

Summary. Blood sera from patients suffering from castorbean-allergy will sensitize the skin of the Philippine crab-eating monkey, *Macaca irus*. The sensitized sites react to castorbean proteins, producing wheals and/or blanching characteristic of similar passive transfer tests (P.-K. tests) in nonallergic human volunteers. Skin reactions in the monkey are more easily interpreted when the colloidal dye Evans Blue is injected intravenously 5 or 10 minutes prior to challenge with the suspected allergenic material. The passive transfer of human serum antibodies into the skin of *Macaca irus* appears to be useful as a method which may be applied in lieu of the Prausnitz-Küstner test (on human volunteers) to avoid the grave risk of infection with serum hepatitis.

The authors wish to acknowledge the cooperation of Dr. Carl Lamanna, Scientific Director, Navy Biological Lab., Univ. of California, in permitting use of his laboratories and animals for preliminary tests. The senior author was assisted in the skin testing on monkeys by Laurence John Layton.

John Kneeland, Director of Research, and Richard Purdy, Assistant to the Director, Pacific Vegetable Oil Co., cooperated in arranging for blood samples from employees appearing to be sensitive to castorbean meal.

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Influence of Goitrogen (Tapazole) in Feed on Thyroxine Secretion Rate of Cockerels.*† (27016)

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In determination of thyroxine secretion rate (TSR) of mature birds, it has been found necessary to block recycling of I^{131} resulting from metabolism of thyroidal I^{131}(1). This is due to fact that apparent thyroidal-I^{131} release rate in many birds is so slow (flat curve) that the blocking action of increasing graded doses of L-thyroxine can not be observed clearly. For this reason, a goitrogen such as thiouracil (0.1-0.2%) or tapazole (0.05% of diet) has been used to prevent uptake of I^{131} by thyroid.

Reineke and Singh(2) reported that estimates of TSR in rats fed thiouracil to block recycling were about 10% higher than those in rats not receiving a goitrogen. Recently, Mellen(3) reported no significant difference in TSR between 0.1 and 0.2% thiouracil in feed of 10-week-old cockerels. Himeno *et al.*(4) suggested that the TSR of chicks fed 0.1% thiouracil increased significantly in comparison with birds fed control diet, and TSR also increased with the length of time that thiouracil was fed.

Baby chicks up to 3 or 4 weeks of age lose thyroidal-I^{131} so rapidly that their TSR can be determined without a goitrogen. On the other hand, use of goitrogen becomes increasingly necessary for older chicks and mature birds.

Since tapazole is routinely used in our laboratory in TSR determinations, TSR was determined in a group of young birds without tapazole, then repeated with graded levels of tapazole in order to determine its effect on TSR.

Materials and Methods. One hundred cockerels of a White Leghorn X New Hampshire cross were housed in room at 60° F. for 2 weeks, then TSR was determined individually starting at 7 weeks of age by method described by Pipes *et al.*(1) except that no goitrogen was used. Birds were than divided into 4 equal groups on basis of individual TSR when 9 weeks of age. TSR determination was then repeated with controls and groups fed 0.025, 0.0375, and 0.05% tapazole added to their ration. When birds were 19 weeks old TSR was determined again using 0.05% tapazole to prevent recycling.

Results. TSR of 100 cockerels without tapazole determined at 7 weeks ranged from 0.5 to 1.5 μg/100 g body weight with mean of $0.89 \pm .02$ μg. Upon repeating TSR at 9 weeks control group (no tapazole) showed a decreased mean of $0.74 \pm .04$ μg/100 g body weight. Groups on tapazole at levels of 0.025, 0.0375, and 0.05% of ration had TSR of $1.11 \pm .08$, $1.18 \pm .08$, and $1.27 \pm .07$ μg/100 g body weight, respectively. TSR of control group (without tapazole) was significantly lower than experimental groups ($P<0.01$). However, mean TSR of birds on graded levels of tapazole were not significantly different although graded in response.

On basis of this comparison, it appeared that use of tapazole partially to completely blocking I^{131} recycling increased apparent TSR from 0.74 to 1.21 μg/100 g body weight or 63.5%. To determine whether such a percentage correction factor could be applied uniformly to birds with increasing TSR, data was reclassified on basis of TSR without tapazole in 7 week old cockerels. Birds with TSR of 0.5 μg without tapazole showed an increase to 1.05 μg or an increase of 110%, birds with TSR of 0.75 μg without tapazole an increase to 1.14 μg or an increase of 52%, birds with TSR of 1.00 μg without tapazole an increase to 1.23 μg or an increase of 23%, whereas birds with TSR of 1.25 μg without tapazole increased to 1.28 μg or 2.4%. Since effect of tapazole in birds with low TSR was very great, whereas birds with high TSR were

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‡ Research Fellow of Atomic Energy Commission of Japanese Government.

unaffected, question of tapazole truly increasing TSR may be raised.

When cockerels were 19 weeks of age, TSR was again repeated using 0.05% tapazole in ration. TSR ranged from .25 to .75 μg/100 g body weight with mean of .45 ± .02, a significant reduction from either 7 or 9 week determination.

Discussion. In determination of TSR in birds and mammals, goitrogens have been employed to block uptake of I^{131} resulting from metabolism of thyroxine-I^{131}. By its use, the true release rate of thyroidal-I^{131} is measured at successive intervals. When thyroxine is then administered at increasing levels to block thyrotropin discharge, the end-point in estimation of TSR is prevention of thyroidal-I^{131} release (at least 95% of previous reading). Goitrogens have been used exclusively in method to increase thyroidal-I^{131} release rate and thus make more obvious the point at which thyroxine level injected has stopped thyroidal-I^{131} release.

No evidence is available which indicates that goitrogens directly stimulate increased TSH secretion when thyroxine is present. Similarly, while goitrogens increase thyroidal-I^{131} release rate, the apparent increased release rate has been believed to be due to prevention of recycling of I^{131}. It is possible, however, it might have such action.

Present data might be interpreted as indicating that tapazole stimulated a 63.5% increase in TSR. However, analysis of data shows that greatest effect was upon birds with low TSR and a progressive reduced effect as TSR increased. If tapazole actually stimulated either increased TSH secretion or thyroidal-I^{131} release, one would expect a similar effect throughout range of TSR.

It is suggested that in 7-week-old birds, TSRs of birds in lower range were underestimated. That is, due to the flatness of thyroidal-I^{131} release curve, it was considered that blocking of release by thyroxine had occurred before it actually had been reached. On basis of present data, underestimation of TSR in birds showing 0.5 μg/100 g body weight without tapazole was about 100%, in those at 0.75 μg was about 50%, in those at 1.0 μg about 25%, whereas those at 1.25 μg were correctly estimated.

If this is correct explanation, need of a level of goitrogen to completely block recycling of I^{131} would be required in all studies of TSR even in young birds and other species with normal rapid release rates. In other words, the role of goitrogens is to increase accuracy of end-point of TSR determination.

Marked decline in mean TSR of these cockerels at 19 weeks with tapazole was quite unexpected since Stahl *et al.*(5) showed in a strain of New Hampshire cockerels only a slight decrease in TSR as sexual maturity was reached.

Summary. Thyroxine secretion rate (TSR) of 100 cross-bred cockerels was determined at 7 weeks of age without tapazole to block I^{131} uptake. At 9 weeks, 4 groups of equal TSR were separated. One group served as a control, whereas other groups were fed 0.025, 0.0375, and 0.05% tapazole in ration. TSR of groups were .74 ± .04, 1.11 ± .08, 1.18 ± .08, and 1.27 ± .07 μg/100 g body weight, respectively. Group not fed tapazole was significantly lower than where included, but differences between tapazole-fed birds were not significant. Tapazole appeared to increase TSR 63.5%. Analysis of these data showed that tapazole increased TSR to greatest extent in groups showing low TSR without tapazole. It is suggested that tapazole does not actually increase TSR but in its absence there is a tendency to underestimate the true TSR due to the slow release rate of thyroidal-I^{131}. It is recommended that in determination of TSR sufficient goitrogen be fed to completely block I^{131} recycling.

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Production of Extracellular Material By Jensen Sarcoma Cells *in Vitro*. (27017)

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Acid mucopolysaccharides and fibers characteristic of connective tissue have been produced *in vitro* by fibroblasts from mature and embryonal tissues(1-5). Mouse lung cells in young cultures were reported to produce collagenase sensitive fibers(6), but this capacity was lost after successive subculture. Abdel-Samie *et al.*(7) found HeLa cells and HLM cells produced species specific albumin in tissue culture. According to Teyssie *et al.*(8), Sarcoma 180 fibroblasts *in vitro* did not produce mucopolysaccharides in quantities detectable by histochemical methods, but small quantities of mucopolysaccharides were demonstrated in the nutritive media by turbidimetric methods. The current study demonstrates the ability of the Jensen sarcoma cells to produce quantities of extracellular material.

Methods. Jensen sarcoma cells were grown in a continuous perfusion system utilizing glass helices as a matrix upon which the cells would adhere. The initial inoculum was 400,000 cells/ml, and the medium used was Medium 5a(9). The initial perfusion rate was 70 ml/24 hrs and subsequent increases in this rate were determined by glucose utilization and pH of the effluent medium. After 9 days of growth the tissue was clearly visible to the naked eye and the cells were harvested. The glass helices with the adherent cell clumps were placed in Bouin's solution at room temperature for 24 hrs. The Bouin's solution was then decanted, and the cell clumps were separated from the helices by agitation in 70% isopropanol. The helices were allowed to settle, and the supernatant containing the cells and extracellular material was removed. The cell aggregates were then further dehydrated through a graded series of isopropanol and embedded in paraffin. Three and 5 μ sections were stained with hematoxylin and eosin, Malloy's trichrome, Lhotka's periodic acid-foot variant(10), Sudan black B, and PAS with diastase digested controls. The methylene blue extinction test, and ninhydrin-Schiff reactions were performed according to the methods outlined by Pearse(11). Other sections were treated respectively with bovine testicular hyaluronidase and streptococcal (chondroitin sulfatase free) hyaluronidase*, 1 mg/ml in phosphate buffer at pH 6.8 for 12 hrs at 37°C. Control sections were incubated in buffer alone. The hyaluronidase treated sections along with their controls were then stained with 1% Alcian Blue in 3% acetic acid(12), Muller's colloidal iron according to the method of Mowry(13), and Toluidine Blue after sulfation(14).

Results and Discussion. The comparison of the Jensen cells as they appeared in stationary T-15 cultures, the perfusion system, and *in vivo* can be seen in Fig. 1. The cells in the perfusion chamber more closely resemble those grown *in vivo* than do the cells cultured in T-15 flasks. They tended to mold to the contour of adjacent cells, overgrow the helices, and form a cloudy gelatinous mass. The consistency of the mass is due in part to extracellular material of 2 types—one consisting of amorphous masses and the other intimately related to individual cells. These differ histochemically.

The amorphous material (Fig. 2), stained with H and E, Mallory trichrome, and reticulum stains, failed to exhibit fibers. This substance stained faintly with PAS and exhibited a sulfation induced metachromasia. These staining reactions were not altered by incubation for 3, 12, and 20 hrs with testicular hyaluronidase or streptococcal hyaluronidase. The staining reaction with PAS was diastase fast. The material did not stain with Sudan B, thereby eliminating sphingolipids; and it did not stain with Alcian Blue or colloidal iron, precluding the possibility of a mucopolysaccharide. The methylene blue extinction was above pH 4, and the ninhydrin-Schiff reaction

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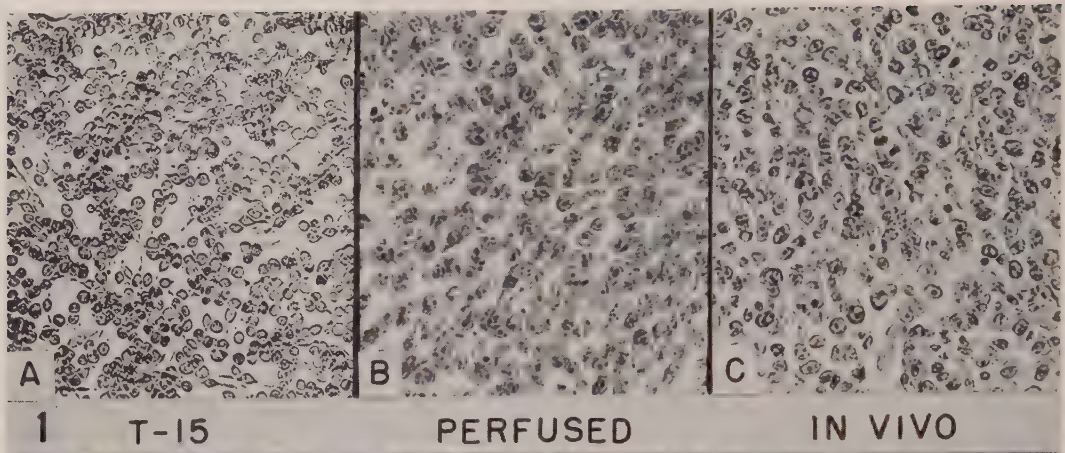
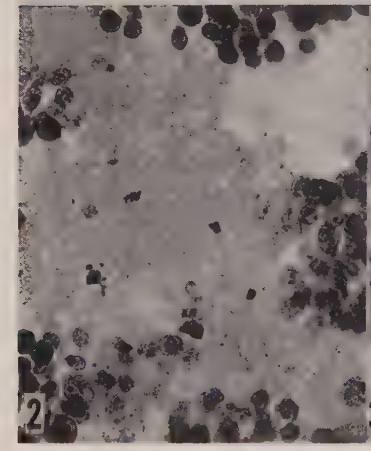
* Streptococcal hyaluronidase was very kindly supplied by Dr. Frederick T. Zugibe, Veterans Admin. Hospital, Pittsburgh, Pa.





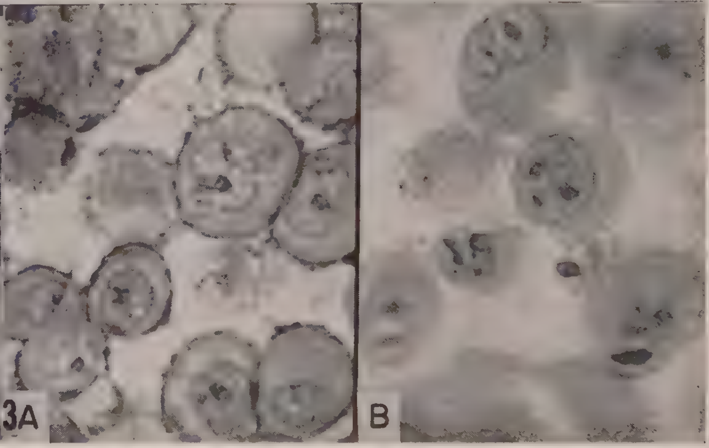


FIG. 1. Comparison of growth patterns of Jensen sarcoma cells grown (A) in the T-15 flask, (B) in a perfusion system, (C) *in vivo*. Note similarity of B and C. (A) Unstained. (B) and (C) Hemotoxylin-Eosin. x112.

FIG. 2. Jensen sarcoma grown in a perfusion system showing masses of amorphous extracellular material. PAS-Hematoxylin. x125.

FIG. 3. Sections of cultures of Jensen sarcoma (A) before and (B) after treatment with streptococcal (chondroitin sulfatase free) hyaluronidase. Colloidal iron-Hematoxylin. (A) - x 840; (B) - x 1120.

was positive. These data suggested the material was a muco- or glycoprotein, a simple protein with serum albumin adsorbed from the media, or a mixture of these substances.

The second type of material which was found in close association with individual cells stained brilliantly with Alcian Blue and with colloidal iron. These staining reactions were removed by 12 hrs incubation with streptococcal hyaluronidase (Fig. 3). This material, therefore, was hyaluronic acid and not chondroitin sulfate.

It is possible that the Jensen sarcoma cells produce small quantities of these materials in T-15 flasks, but it is not readily demonstrable because of the small amount of cellular material produced. A similar situation was reported to occur in Sarcoma 180 cells(8). The Jensen sarcoma cells, unlike some neoplasms, apparently do not produce hyaluronidase(15); therefore, hyaluronic acid must accumulate around the cells.

Summary. Jensen cells grown in a continuous perfusion system produce extracellular material of two types. One is a muco- or glycoprotein or possibly a simple protein; the other, hyaluronic acid.

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Kidney Enzymes of Gluconeogenesis, Glycogenesis, Glycolysis and Direct Oxidation.* (27018)

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Studies of the past two decades demonstrated that the kidney carries out manifold metabolic functions of primary physiologic importance in addition to urine production. The various metabolic contributions of the kidney have been reviewed(1). One of the striking properties of kidney, shared only by liver, is its capacity to produce sugar. Earlier studies involving glucose measurement in arterial and renal vein and recent work utilizing kidney slice incubation techniques to demonstrate net synthesis of carbohydrate were discussed(1,2). More recent investigations on the alternate pathways of glucose metabolism of kidney employed labeled glucose or pyruvate and conclusions were reached on the behavior of metabolic pathways by determination of metabolites and end products of the various pathways(1,2). Thus, it has become of interest to obtain detailed information regarding the activities and behavior of the enzyme systems involved in glucose metabolism in this tissue.

The present work describes the carbohydrate enzymes involved in gluconeogenesis, glucose production, glycogenesis, glycolysis and direct oxidative pathway in the kidney. The enzymes studied are responsible for channeling glucose-6-phosphate into four alternate pathways: glucose-6-phosphatase (glucose release); phosphoglucomutase (glycogen synthesis); phosphohexose isomerase (glycolysis); and glucose-6-phosphate and 6-phosphogluconate dehydrogenases (direct oxidative pathway). The activities of other enzymes of gluconeogenesis and glycolysis (fructose-1,6-diphosphatase and lactic dehydrogenase) are also given. These carbohydrate metabolizing enzymes were studied in the kidney cortex of rats and values were compared with activities found in the liver. The investigation revealed a distinct enzyme pattern in kidney cortex which can be correlated with the behavior of metabolic pathways in this organ as elucidated by isotope approaches.

Materials and methods. Male Wistar rats of 250 g of weight were maintained on Purina Laboratory Chow and water *ad libitum.* The animals were stunned, decapitated and exsanguinated. Kidneys were quickly removed, placed in beakers standing on cracked ice, chilled for 5 minutes and sliced in two. The kidney cortex areas were removed, placed in ice cold beakers, minced with scissors and 10% homogenates were prepared in isotonic KCl solution. Homogenization was carried out with a teflon plastic pestle turning at about 600 r.p.m. for exactly 90 seconds. The supernatant fluid was obtained by centrifuging the tissue homogenates at 100,000 g for 30 minutes at 0°C. A refrigerated Spinco model L centrifuge was used.

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Cell counts and biochemical assays. The cellularity of the kidney was determined by counting the cell nuclei in cortex homogenates following previously described procedures employed in counting liver nuclei(3). Nitrogen content was determined by the micro-Kjeldahl procedure. Glucose-6-phosphatase activity was measured in the homogenate(4,5), and all other enzyme activities were assayed in the supernatant fluid. Phosphoglucomutase activity was assayed according to Najjar(6); phosphohexose isomerase, glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities were followed by the methods of Glock *et al.* (7) and Glock and McLean(8). Fructose-1,6-diphosphatase and lactic dehydrogenase were measured according to Weber and Cantero(9). All enzyme assays were carried out at 37°C under linear kinetic conditions. Activities are expressed per average cell.

Results and discussion. A comparison of the cellularity, nitrogen content and carbohydrate enzyme activities in the average cell of rat liver and kidney is presented in Table I. The cellularity of the kidney was 58% higher than that of the liver. However, nitrogen content of the average kidney cell was about one-half of that of the average hepatic cell.

The *glucose-6-phosphatase* activity was 74% of that found in the liver which is in good agreement with previous reports(5,10). The *fructose-1,6-diphosphatase* activity was approximately one-third of the liver activity. The presence of these 2 specific phosphatases is necessary for the functioning of the gluconeogenic processes which were demonstrated by

TABLE I. Comparison of Carbohydrate Enzyme Activities.*

	Liver		Kidney	
Cellularity‡	178 ± 5	(100)†	281 ±20	(158)
Homogenate nitrogen§	1.7 ± .2	(100)	.94 ± .1	(55)
Supernatant " §	.9 ± .02	(100)	.47 ± .06	(52)
Phosphohexose isomerase‖	685 ±66	(100)	811 ±58	(118)
Lactic dehydrogenase	593 ±19	(100)	140 ±14	(24)
Phosphoglucomutase	127 ±11	(100)	2.8 ± .2	(2)
Glucose-6-phosphatase	36.3 ± 2.1	(100)	26.9 ± 4.9	(74)
Fructose-1,6-diphosphatase	26.5 ± 2.8	(100)	8.3 ± 1.4	(31)
Glucose-6-phosphate dehydrogenase	4.4 ± .4	(100)	.3 ± .03	(7)
6-Phosphogluconate dehydrogenase	12.3 ± .8	(100)	1.0 ± 0	(8)
		Ratios		
Phosphohexose isomerase/ Phosphoglucomutase	5.4	(100)	290	(5370)
Glucose-6-phosphatase/ Glucose-6-phosphate dehydrogenase	8.3	(100)	90	(1084)

* Mean values and standard errors represent 4 or more rats in each group.

† Data expressed in %. Liver values taken arbitrarily as 100%.

‡ In millions of nuclei counted/g wet weight of tissue.

§ Calculated as mg nitrogen/cellularity x 10^8.

‖ Enzyme activities given per cell x 10^7 μmoles of substrate metabolized/hr at 37°C.

pyruvate to glucose conversion utilizing isotope methods(1,2). *Phosphohexose isomerase* was in the same activity range in liver and kidney. The function of this powerful enzyme which channels glucose-6-phosphate into glycolysis is in line with the presence of glycolysis described in this tissue(1,2). *Lactic dehydrogenase* activity was less than one-third of that in the liver; however, it was shown that even an activity of less than 20% for this very active enzyme is compatible with production of large amounts of lactate(9,11). The presence of *glucose-6-phosphate* and *6-phosphogluconate dehydrogenases* indicates the capacity of this tissue to carry out direct oxidation of glucose-6-phosphate, channeling it into pentose production. However, since these dehydrogenases are less than 10% of activities found in the liver it appears that the direct oxidative pathway probably contributes comparatively little as an alternate metabolic pathway of glucose-6-phosphate metabolism. A similar low activity in the kidney cortex was noted for *phosphoglucomutase* activity which was less than 5% of the enzyme levels observed in liver. Experience obtained in the study of behavior of phosphoglucomutase in Novikoff hepatoma and Morris hepatoma 5123(12) as well as in adipose tissue(13) indicates that when this enzyme occurred in activities of less than 15% of those found in liver poor glycogenesis existed. This is in line with the low glycogen deposition in kidney(1,2,10) in comparison with the powerful glycogenic processes of the liver which has very active phosphoglucomutase activity. The difference in this glycogenic enzyme activity may explain the fact that cortisone administration results in glycogen deposition in liver(10) which contains high phosphoglucomutase activity, but not in kidney (2,14) where this enzyme activity is very low. On the other hand, cortisone administration resulted in increased gluconeogenesis in both liver and kidney(2,10,14), which may be explained by the similarity on the enzymatic level of the presence of glucose-6-phosphatase and fructose-1,6-diphosphatase in these 2 organs.

The predominance of the capacity of certain enzymes involved in channeling of glucose-6-phosphate into alternate metabolic pathways is shown by the ratios of these enzymes given in Table I. The ratio of phosphohexose isomerase/phosphoglucomutase compares the 2 enzymes which channel glucose-6-phosphate into the energy-yielding (glycolytic) and storing (glycogenic) pathways and the glucose-6-phosphatase/glucose-6-phosphate dehydrogenase compares the activities of the 2 enzymes which channel glucose-6-phosphate into hydrolysis (glucogenesis) or into pentose production (direct oxidative pathway). The phosphohexose isomerase/phosphoglucomutase ratio is 5.4 in the liver, but is 290 in the kidney. The glucose-6-phosphatase/glucose-6-phosphate dehydrogenase ratio is 8.3 in the liver; 90 in the kidney. These ratios indicate that in kidney cortex the enzyme capacity for channeling glucose-6-phosphate into glycolysis and glucose production markedly predominates over the enzymes involved in channeling this hexose ester into glycogenesis or pentose production.

The behavior of kidney carbohydrate enzymes under various physiologic and pathologic conditions is being studied.

Summary. The present work described the cellularity, nitrogen content and kidney enzymes involved in gluconeogenesis, glucose production, glycogenesis, glycolysis and direct oxidative pathway in kidney cortex. The results were compared with values found in the liver. The kidney contained 50% more cells per unit weight; however, the average kidney cell contained only about half the nitrogen content found in the liver cell. These studies demonstrated that the enzyme systems involved in glycolysis and gluconeogenesis, phosphohexose isomerase, lactic dehydrogenase, glucose-6-phosphatase and fructose-1,6-diphosphatase, are present in the kidney cortex at relatively high levels. On the other hand, the low activities for kidney phosphoglucomutase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase suggest that this tissue has poor glycogenic and direct oxidative pathways in comparison with the metabolic pathways of the liver. This investigation revealed a distinct carbohydrate enzyme pattern in kidney cortex and showed that the activities of these enzymes as determined in tissue homogenate and supernatant fluid can be well correlated with studies on the relative importance of glucose metabolic pathways as elucidated by

isotope techniques on kidney slices.

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Distribution of Cortical "Feedback" Fibers in the Nuclei Cuneatus and Gracilis.* (27019)

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(Introduced by F. H. J. Figge)
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In cat and monkey pyramidal fibers are distributed to the nuclei cuneatus and gracilis (1,2,3,4). The fiber distribution within these nuclei displays some degree of organization (3,4) which has been investigated further.

Experiments. A. In 2 cats the rostral pole of one hemisphere was removed, including the sensory-motor areas. Using the Nauta-Gygax impregnation technique(5), the degenerating cortical fibers were found to pass as recurrent bundles through the basal parts of the nucleus cuneatus, as previously reported(3). Similar bundles pass alongside the nucleus gracilis. At caudal levels, these degenerating fibers are distributed to the basal parts of the nucleus cuneatus (and gracilis) (Fig. 7,8). However, only few fibers are distributed to the characteristic dorsal cell clusters (Fig. 2, 3, 6, 7, 8). The fiber distribution in both the nuclei becomes more diffuse in passing from a few millimeters behind the obex rostrad. Some of the longitudinal fibers in the basal parts of the nucleus cuneatus move dorsally towards the surface (Fig. 6), thus cutting through the area of the cell clusters. This narrow fiber pathway widens rostrally at the expense of the clusters, resulting in a more widespread fiber distribution (Fig. 1, 4, 5). However, at rostral levels, some clusters persist, e.g., in the ventrolateral parts of the nucleus cuneatus and along its dorsal medial border (Fig. 5).

The cyto- and dendritic architecture of the nuclei, in Nissl and Golgi material (6-weeks-old kittens impregnated following Moliner(6)), likewise displays rostro-caudal differences, described by Cajal. The caudal cell clusters contain round cells, possessing short, repeatedly ramifying dendrites (Fig. 10, 11, 12). The diameter of the dendritic territories equals approximately 10 times that of the cell body. The dendrites in one cell cluster frequently interlace. The question arose whether this phenomenon could be related to the mutual inhibition characteristic for the cells at these levels (13). In the tail end of the nucleus gracilis these cells are more triangular, possessing longer dendrites (Fig. 13). The basal cells in the caudal part of the nucleus cuneatus are

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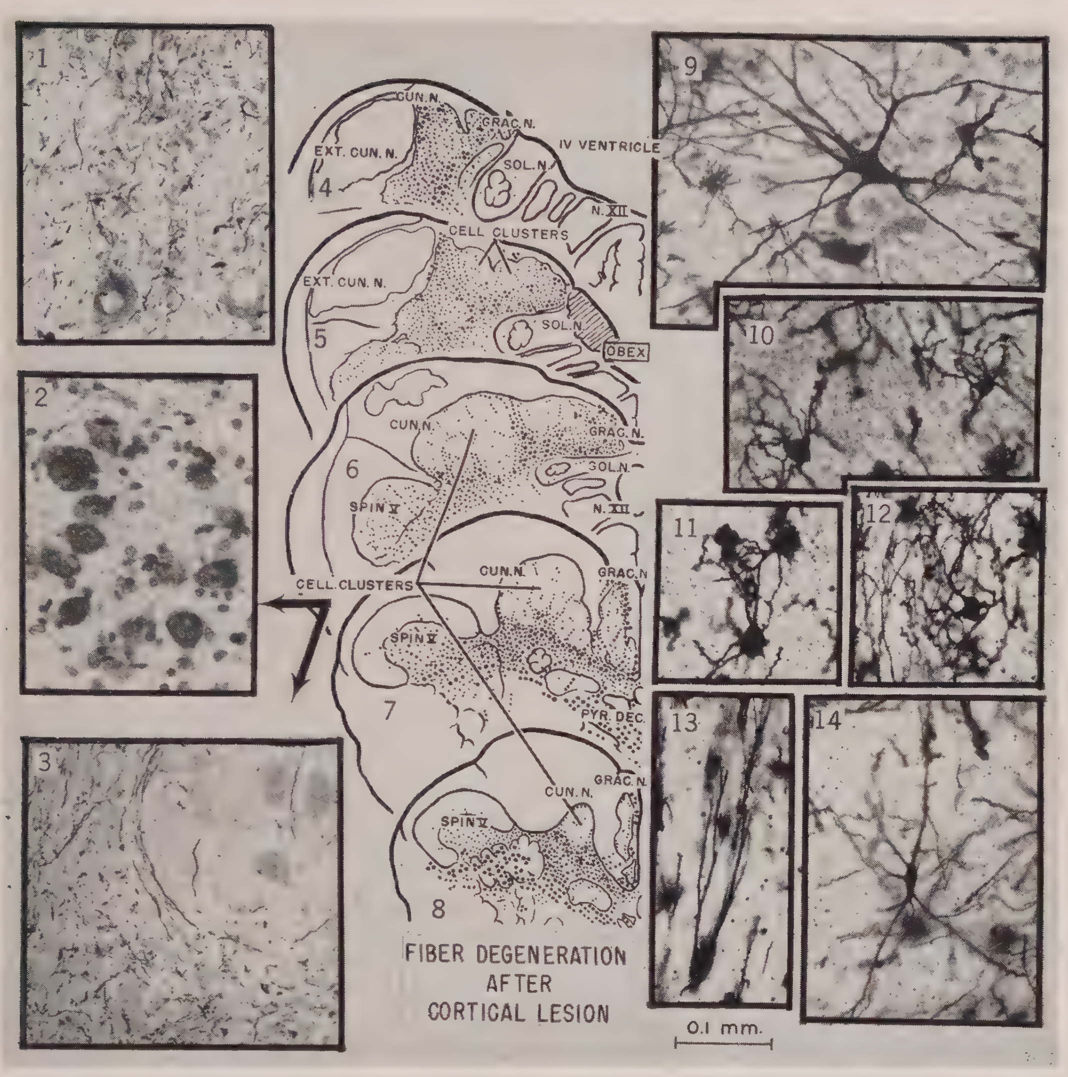
† Research Assistants in Dept. of Anatomy, Summer of 1960.



FIGS. 1 and 3. Fiber degeneration in rostral and caudal parts of nucleus cuneatus respectively. (Nauta-Gygax ±400X, and ±600X).

FIG. 2. Cell cluster in caudal part of nucleus cuneatus (cresyl violet, ±500 X).

FIGS. 4-8. Semidiagrammatic representation of fiber degeneration in nuclei cuneatus and gracilis, following lesion of the contralateral hemisphere. .˙. degenerating bundles, ... degenerating fibers.

FIG. 9. Large multipolar cell in rostral part of nucleus cuneatus. (Golgi Cox).

FIGS. 10, 11, 12. Cells from cell clusters of nucleus cuneatus.

FIG. 13. Cell in tail end of nucleus gracilis.

FIG. 14. Cell taken from caudal base of nucleus cuneatus.

fusiform and triangular in shape, possessing long sparsely ramifying dendrites (Fig. 14). Somewhat similar cells are found within the nucleus gracilis and along the surface of both nuclei. Rostrally, the cell clusters largely are replaced by triangular and multiplolar cells (Fig. 9) possessing long, sparsely ramifying dendrites. The diameter of their dendritic territory is approximately 5 times larger than in the caudal clusters. Smaller cells are scattered between the multipolar cells (Fig. 9). The few rostral cell clusters resemble the caudal ones, but the cells seem smaller and the dendritic trees less dense.

A comparison of the fiber distributions revealed that the cortical fibers are distributed primarily to those parts of the nuclei which contain triangular, fusiform and multipolar cells possessing long sparsely ramifying dendrites. The fiber distribution within these parts of the nuclei frequently seems to coincide with the orientation of the sprasely ramifying dendrites. However, only a few cortical fibers are distributed to the cells with small and frequently ramifying dendrites, present primarily in the cell clusters.

B. To judge the projection of the nuclei to the thalamus, the ventrolateral mesencephalon (medial lemniscus) was transected in 4 kittens at the age of 2 weeks. Following survival periods ranging from 2 to 16 weeks, many of the round cells in the caudal clusters—receiving very few cortical fibers—displayed only a nucleus with or without a thin rim of cytoplasm (Nissl stain). However, some of the basal cells in the caudal parts of the nucleus cuneatus and some of the rostral multipolar and triangular cells—receiving many cortical fibers—were less conspicuously affected. Their cell bodies were somewhat smaller, but some of them displayed normal Nissl substance. The smaller rostral cells were more severely affected.

Similar changes were observed in retrograde Golgi studies, in which the mesencephalic lesion at the age of 2 weeks was followed by Golgi impregnation at the age of 6 weeks. The caudal clusters were severely affected. Many of the impregnated cells were small, possessing thin dendrites. Their dendritic ramifications appeared more numerous than on the normal side, owing to sprouting or to better encrustation. Some of the rostral triangular and multipolar cells were less severely affected.

These findings suggest that the cells in the caudal clusters maintain less collaterals, uninterrupted by the lesion, than some of the triangular and multipolar cells in the caudal base of the nucleus cuneatus and in the rostral part of both nuclei. However, in many of the animals a few of the most medial bundles of the medial lemniscus carrying fibers from the rostral part of the nuclei(7), were spared. This renders the suggested differences in collateralization less than convincing. Nevertheless, such differences may exist, for in one kitten and in one adult cat a total transection of the medial lemniscus resulted in a somewhat similar distribution of retrograde cell reaction. Obviously, this point requires further investigation.

Discussion. The distribution of the pyramidal fibers as seen within the nuclei cuneatus and gracilis corroborates macroelectrode findings(8) which demonstrated that in the caudal parts of the nucleus cuneatus the pyramidal influence is exerted primarily on basal cells. On the other hand, microelectrode findings (9) indicate that a pyramidal influence is exerted also on the dorsally located cell clusters. The sparsity of the cortical fibers in these cell clusters suggests that this influence in part may be exerted indirectly, via other neurons. This might be suggested also by Waller's microelectrode findings(10) if they were obtained in caudal parts of the nucleus cuneatus.

The differences in dendritic architecture between dorsal cell clusters and basal cells in the caudal parts of the nucleus cuneatus seem to parallel differences in function. This is suggested by the fact that the dorsal cells—with short, frequently ramifying dendrites—apparently maintain small, cutaneous receptive fields located distally, while the basal cells—with long, sparsely, frequently ramifying dendrites—apparently maintain larger cutaneous receptive fields located proximally(11). These dorso-ventral differences in dendritic architecture within the caudal parts of the nucleus cuneatus resemble the rostro-caudal differences within the nuclei. The latter likewise may parallel differences in function. This is suggested by the fact that the rostral cells, many of which have long, sparsely ramifying dendrites, in general appear to maintain larger cutaneous receptive fields than the cells in the caudal clusters(13) and in part appear to transmit different sensory information(12, 14). In other words, this comparison suggests a possible correlation between the dendritic architecture of the cells and the size of their receptive fields, exemplified by the fact that the cells of Fig. 10, 11, 12, Fig. 13 and Fig. 9, respectively are found in the parts of the nuclei containing cells with increasingly larger receptive fields(13).

If these inferences are correct, the cortical fibers are distributed primarily to cells with low resolution power (large receptive fields), while only a few cortical fibers are distributed to cells with high resolution power (small receptive fields). Therefore, the question arises whether the function of the cortical feedback might be related to the localizing resolution power of this sensory system.

Summary. The distribution of the cortical feedback fibers in the nuclei cuneatus and gracilis has been compared with the cyto-architecture and the dendritic architecture of these nuclei. This comparison revealed that the cortical fibers distribute primarily to those parts of the nuclei containing multipolar, triangular, and fusiform cells maintaining long, sparsely ramifying dendrites. The cells with frequently ramifying dendrites, on the other hand, receive only few fibers. Furthermore, preliminary evidence suggests that some of the former cells maintain more collaterals than the latter.

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Effect of Fat-Free Diets and Lipid Unsaturation on Rat Tissue Cholesterol Levels.† (27020)

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The degree of unsaturation of dietary fats and oils has been postulated as a factor which may influence cholesterol metabolism(1). Variations in degree of unsaturation have been commonly obtained by either partial hydrogenation of a given lipid or by the combination of lipids from different sources. Although such manipulation yeilds a lipid with a specific degree of unsaturation as defined by iodine value, the effect of positional and geometrical isomers produced by partial hydrogenation, and of short-chain fatty acid triglycerides, cannot be evaluated. Thus the effect on liver and blood cholesterol levels produced by a lipid from a single source, but differing in degree of unsaturation, is of interest. Three degrees of unsaturation were obtained by use of unhydrogenated oil, an aliquot of the same oil completely hydrogenated, and a simple mixture of these in equal proportion. Data for both corn oil and safflower oil are presented.

Methods. An experimental group of 8 male rats* each weighing ca. 100 g was housed 2 rats per metal, screen-bottom cage. Rats were allowed access to experimental diet and water *ad libitum.* Composition of the basal diet is given in Table I. Addition of 5% lipid and/or 2% cholesterol was made at the expense of potato starch. Lipids with different degrees of unsaturation, as determined by iodine number, were obtained by use of (a) commercially

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‡ né M. Korzenovsky.

* Carl Wilson and Sons, Beech Grove, Ind.

TABLE I. Composition of Basal Diet.

Component	%
Lipid[1]	0
Cholesterol	0
Casein, vitamin-free[2]	20.0
Potato starch	37.0
Sucrose	28.0
Cellulose, powdered	10.0
Salt mixture[3]	4.4
ρ-aminobenzoic acid	.1
Inositol	.4
Choline	.1
Vitamin mixture[4]	+

[1] Dietary lipids were those described in Methods.

[2] Borden Co., Chicago.

[3] Hawk-Oser salt mixture(5).

[4] The vitamin mixture supplied the following in mg/100 g of diet: riboflavin, .04; pyridoxine·HCl, .02; niacin, .64; folic acid, .002; biotin, .0016; vit K diphosphate, .04; α-tocopherol, .80; ascorbic acid, .50; calciferol, .036; vit A, .443.

TABLE II. Effect of Dietary Additions on Plasma Cholesterol Levels.[1]

Dietary Lipid	Without Cholesterol	With 2% Cholesterol
	w/o Lipid	
—	44 ± 5.5	43 ± 2.6
	w/Corn oil	
Sat.	56 ± 2.7	88 ± 10.5
1:1	68 ± 3.7	77 ± 6.4
Unsat.	61 ± 3.8	80 ± 3.6
	w/Safflower oil	
Sat.	52 ± 3.2	80 ± 9.9
1:1	66 ± 4.3	125 ± 11.0
Unsat.	87 ± 4.6	99 ± 5.8

[1] Data expressed as mg/100 cc plasma ± stand. error.

available oil, (b) the same oil, but completely hydrogenated**, and (c) 1:1 mixture of a and b. By this method iodine numbers(2) of 130, 1.5, and 61 for corn oil***, and 137, 1.5, and 57 for safflower oil**** were obtained. The hydrogenated lipid was found to be free of *trans*-isomers by the method of Holmes, Ener, and Edmondson(3). Animals were sacrificed after 8 weeks on experimental diets. Terminal blood was obtained by cardiac puncture and was heparinized to obtain plasma; also, the liver was removed. Liver and plasma were frozen and stored at −15°C in tightly sealed bottles until analyzed. Hepatic free and total cholesterol and plasma total cholesterol were determined by the method of Sperry and Webb(4).

Results. Plasma total cholesterol levels were lowest and were independent of the cholesterol content of the diet when rats were fed a lipid-free diet (Table II). However, addition of 5% lipid to the diet increased plasma cholesterol levels. The greatest increase in total cholesterol was observed when 2% cholesterol was added to lipid-containing diets. There was no apparent correlation between plasma cholesterol levels and degree of unsaturation in corn oil-fed rats. Plasma cholesterol levels obtained with saturated safflower oil, without or with 2% cholesterol, were not different from levels observed in corresponding groups fed saturated corn oil. However, plasma cholesterol levels were higher using either unsaturated safflower oil without cholesterol or the 1:1 mixture and unsaturated safflower oil with cholesterol than in the respective corn oil-fed rats.

When a cholesterol-free diet was fed, hepatic free and total cholesterol concentrations were not affected by either the concentration or characteristics of the dietary lipid (Table III). However, when 2% cholesterol was added to a diet which was either lipid-free or contained 5% lipid in the diet, free and total cholesterol concentration was increased. Hepatic free cholesterol concentration was independent of amount and degree of unsaturation of dietary lipid when cholesterol was fed. However, total hepatic cholesterol level was determined by amount and degree of unsaturation of the dietary lipid. In rats fed 2% cholesterol in the diet, the smallest increase in hepatic cholesterol was observed with a lipid-free diet. Highest cholesterol concentrations were observed in rats which were fed diets containing 5% lipid and 2% cholesterol. In addition, total cholesterol concentration was dependent upon degree of unsaturation of dietary lipids. An increase in esterified cholesterol characterized the increase of total hepatic cholesterol concentration. The esterified cholesterol fraction was dependent upon the amount and degree of

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** Hydrogenation was carried out in a stainless steel hydrogenator in presence of Rainey nickel. The reaction was essentially complete at the end of 3 hours; however, it was allowed to proceed until pressure within the reaction vessel had equilibrated at 500 p.s.i. The product was discharged and filtered at 100°C.

*** Mazola Oil, Corn Products Co., Argo, Ill.

**** Holmes, Welch & Clark Co., New York.

TABLE III. Effect of Dietary Additions on Hepatic Cholesterol Levels.[1]

Dietary Lipid	Without Cholesterol		With 2% Cholesterol	
	FC[2]	TC[2]	FC	TC
		w/o Lipid		
—	1.77 ± .07	1.99 ± .04	2.53 ± .18	3.30 ± .25
		Corn Oil		
Sat.	1.86 ± .06	2.04 ± .07	2.49 ± .11	5.07 ± 1.13
1:1	1.72 ± .25	1.88 ± .05	2.55 ± .08	11.70 ± 2.19
Unsat.	1.90 ± .09	2.17 ± .10	2.56 ± .15	12.90 ± 2.60
		Safflower Oil		
Sat.	1.90 ± .08	2.22 ± .04	2.72 ± .07	4.66 ± .59
1:1	1.55 ± .08	2.23 ± .06	2.73 ± .08	14.77 ± 2.97
Unsat.	1.99 ± .10	2.35 ± .13	2.28 ± .09	21.10 ± 3.15

[1] mg/g ± standard error.

[2] FC and TC are free and total cholesterol respectively.

saturation of the dietary lipid.

Discussion. In this study greater tissue cholesterol concentrations were seen when cholesterol was fed, indicating a greater rate of absorption than of excretion of the sterol nucleus in attainment of the steady state. Hepatic cholesterol responded more sensitively than did plasma cholesterol to qualitative and quantitative changes of dietary lipid. These data support the hypothesis that in the rat, liver reflects changes of cholesterol metabolism more sensitively than does the plasma(6). The increase in hepatic cholesterol was primarily confined to esterified cholesterol which accumulates with an excess of either exogenous or endogenous cholesterol in the animal body(7,8,9). In either rabbit or chicken a greater increase of the free cholesterol fraction is observed than in the rat.

Dietary lipid has been deemed necessary for absorption of cholesterol(10). The results reported here indicate that dietary lipid was not obligatory for cholesterol absorption. Inasmuch as hepatic cholesterol levels were greatly increased upon addition of lipid to the diet, endogenous lipids in the intestine were not present in sufficient concentrations for maximal cholesterol absorption. Similar observations have been made for the rat(11,12) and the chicken(13,14,15).

There have been many reports on the effect of unsaturation of dietary lipids on tissue cholesterol levels(1). Generally it has been concluded that a cholesterol-lowering effect of lipids was dependent upon either unsaturation or certain essential fatty acids. However, we observed lowest hepatic cholesterol levels when a completely hydrogenated lipid was fed. Hepatic cholesterol levels were directly related to increased unsaturation of the dietary fat. Likewise, Dam *et al.*(13,15) reported that chickens had higher cholesterol levels when fed unsaturated, rather than partially saturated, peanut oil. Later, this group(14) found no difference in hepatic cholesterol content of chickens when they were fed either hydrogenated or unhydrogenated peanut oil with cholesterol. Beare *et al.*(16) found that rabbit hepatic cholesterol concentrations were directly related to unsaturation of dietary lipid. Although there was no obvious correlation between total unsaturation and plasma cholesterol, plasma cholesterol levels were higher in safflower-oil-fed rats than in corn oil-fed rats.

It is interesting that safflower oil is richer in linoleic acid than is corn oil. That a dietary fat rich in linoleic acid increased serum and hepatic cholesterol levels in the rat is in agreement with results of Funch(17) and of Klein (9). In addition Gerson et al.(18) found that total body cholesterol of the rat was significantly greater with linoleic acid than when beef fat was fed.

Since corn oil and safflower oil consist almost entirely of triglycerides containing saturated and unsaturated fatty acids with 18 carbon atoms, the completely hydrogenated lipids are composed primarily of stearic acid triglycerides. Inasmuch as the highest hepatic cholesterol levels were observed when cholesterol was fed with unsaturated lipid, it may be concluded that unsaturated fats increase rate of cholesterol absorption. Mattson(19) has demonstrated that absorbability of a fat was directly proportional to its content of triglycerides composed of unsaturated fatty acids with a chain length of 18 carbon atoms or more. Absorption of dietary cholesterol may be dependent upon fatty acid composition of the dietary lipid because constituent fatty acids affect digestibility of the dietary lipid.

Fatty acids may affect cholesterol absorption by some mechanism other than by altering lipid digestibility. Cholesterol absorption has been associated with pancreatic cholesterol esterase, which catalyzes the esterification of cholesterol with fatty acids(20). The esterification of cholesterol *in vitro* proceeds at a much greater rate with unsaturated acids(21); the same relative rates presumably would hold *in vivo*. Indeed it has been shown that feeding stearic acid resulted in very poor cholesterol absorption(22,23,24). Funch *et al.*(17) observed that rats fed margarines containing hydrogenated whale oil have lower endogenous blood cholesterol levels than rats fed butter.

Summary and Conclusion. 1. Rats were fed diets which were either fat-free or contained 5% lipid and either without or with 2% cholesterol. Dietary lipids with 3 degrees of unsaturation were obtained by use of unhydrogenated oil, completely hydrogenated oil, and a 1:1 mixture of the two. 2. A dissociation of plasma and hepatic cholesterol was observed. Liver reflected changes in cholesterol metabolism more sensitively than did plasma. 3. Hepatic cholesterol concentrations were directly related to degree of unsaturation of dietary lipid when cholesterol was fed. The increase in hepatic cholesterol was confined to esterified cholesterol. 4. Dietary lipid was not obligatory for cholesterol absorption. However, absorption appeared to be dependent upon digestibility and fatty acid composition of dietary lipid.

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Serum Protein and Calcium of Pigeons During The Reproductive Cycle.† (27021)

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Advantages in the use of pigeons for certain hormonal and metabolic studies(1) make desirable additional information concerning the serum proteins of this species, especially as related to sex and reproduction. Plasma or serum proteins have been studied electrophoretically during the menstrual cycles of the baboon(2) and of women(3). However, inasmuch as this technique has not been used in following such changes in other species, its application to studies of the reproductive cycle of the female pigeon(4-6) seemed desirable. To this end, serum electrophoretic patterns of both male and female birds have been obtained, together with the accompanying values for serum total protein and calcium, in the course of the egg-laying cycle.

Materials and Methods. Adult white Carneau pigeons, labeled as to sex, were purchased from the Palmetto Pigeon Plant, Sumter, S. C. They were housed in separate pairs, male and female, in outdoor cages. The birds were fed and watered twice a day, with supplies available *ad libitum.* The feedings, separated by an interval of 4 hours, consisted of a diet of a 1:1 mixture of scratch (wheat, corn, oats), and peas, with a mineral mixture supplement. Eight pairs of mated males and females were used. At various points of the reproductive cycle, which were determined as outlined by McDonald and Riddle(6) and noted below, 8 to 12 ml samples of blood were withdrawn from a wing vein as quickly as possible just prior to feeding. The blood was allowed to clot, and centrifuged. The separated serum was then kept at refrigerator temperature for use in analyses. Total nitrogen was determined gasometrically, according to Van Slyke and Kugel (7). Calcium was determined according to Sendroy by ashing(8), and spectrophotometric analysis at 400 mμ(9). For electrophoretic analysis, 1 ml samples of serum were diluted with 2 ml of a buffer solution of 0.02 M PO_4 (1:5 of NaH_2PO_4, and Na_2HPO_4, at pH 7.4) and 0.15 M NaCl(10). Following overnight dialysis against 500-1000 ml of the same buffer solution, analysis was carried out in a Perkin-Elmer apparatus (schlieren scanning, in a standard 2 ml two channel cell, 15 mm path, 2 mm width, 18.0-18.7 ma current) at 2°C. Although both ascending and descending patterns were obtained, only the latter were used for evaluation of the distribution of the proteins by the areametric method of Sendroy and Cecchini (11).

From each of the 16 birds, 5 samples of serum, corresponding to points designated in the following outline of a reproductive cycle, were obtained. The first day of a cycle was marked by the laying of the first egg of a clutch. Sample 1 was taken on the following day, preceding the laying of the second egg on the third day. Sample 2 was taken 3 days after appearance of the second egg. Sample 3 was taken 5 days before the estimated hatching date, or about the 14th day of the cycle. Sample 4 represented a point in the cycle 2 days after the first hatching (when crop milk formation was maximal). Sample 5 was taken 11 days later (when crop milk production was considerably decreased), several days prior to onset of a new cycle. The cycles reported here were observed throughout the month of November.

Results. As representative of changes common to all of the birds studied, Fig. 1 gives electrophoretic patterns of the sera of a mated pair of pigeons at various points in the reproductive cycle. The female patterns shown are (A) at ovulation (Sample 1), (B) in the subsequent incubation period prior to crop gland enlargement (Sample 2), and (C) at approximate hatching date (Sample 4). The male patterns (approximately the same for all 5 samples) shown are (D) for Sample 1, and (E) for Sample 4.

Average mobilities of the several serum components for male and female pigeons are given

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† The authors are indebted to Dr. Joseph J. Martorano, for bleeding the pigeons used in this study.

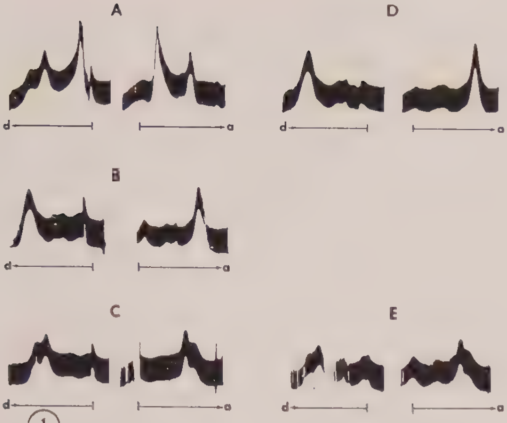
TABLE I. Mobilities and Percentage Distribution of Proteins from Electrophoretic Analysis of Sera of Pigeons at Selected Points of Reproductive Cycle.

Protein	Globulin						Albumin		"F"	
	γ-		β-		α-					
Sex	F	M	F	M	F	M	F	M	F	M
Mobility, x $10^{-5}cm^2Volt^{-1}sec^{-1}$										
No. of Sera	33	20	34	21	33	21	34	21	26	11
Mean	.5	.4	2.1	2.1	3.6	3.5	4.4	4.4	5.3	5.1
% of Total Protein										
Sample 1										
No. of Birds	8	6	8	6	8	6	8	6	7	2
Mean	14	11	40	16	11	14	30	50	6	9
Sample 2										
No. of Birds	8	3	8	3	8	3	8	3	3	1
Mean	15	15	21	20	9	10	44	43	11	12
Sample 3										
No. of Birds	7	7	7	7	7	6	7	7	7	3
Mean	12	10	13	18	12	10	33	49	30	14
Sample 4										
No. of Birds	7	3	7	3	7	3	7	3	6	3
Mean	16	14	14	16	13	11	34	32	23	28
Sample 5										
No. of Birds	5	4	5	4	5	4	5	4	4	3
Mean	13	16	22	15	9	13	31	34	25	23

in Table I. The mobilities of 0.5, 2.1, 3.6, 4.4, and $5.2 \times 10^{-5}cm^2Volt^{-1}sec^{-1}$, respectively, obtained for γ-, β-, and α-globulins, albumin, and an "F" component (discussed below), are in good agreement with the data for a single sample of pigeon serum in pH 7.4 phosphate given by Moore(10). Also shown in Table I are averages of the percentage distributions of the components for the 5 points of the cycle, arbitrarily identified and classified on the basis of their mobilities. Table II gives averages of serum total protein values for the cycle points, with corresponding values for β-globulin and total calcium.

Discussion. Visual comparison of the patterns (Fig. 1) indicates a marked change in protein composition of the female pigeon serum during the reproductive cycle, with relatively little change in the male pattern. The quantitative accounting of the distribution in Table I indicates relatively unchanged γ- and α-globulin throughout all samples, and a large increase in β-globulins (also reflected in total protein values of Table II), of female serum at time of ovulation (Sample 1). For the post-ovulation Sample 2, however, the female pattern (B) and distribution values closely resembled those of the normal male (D). Matching of the patterns of the sexes also occurred (with small but notable change from normal in the male serum (E)) of Sample 4. Present in many of the samples, at all 5 cycle points for both female and male, was a component which moved faster than the albumin. This component "F" is a pre-albumin or a protein possibly related to the "f" component observed in the serum of several species of birds(10,12), and especially of the chicken(13-15), by workers who did not follow changes throughout a cycle. The component "F" was increased in relative concentration in Samples 3, 4 and 5 for the female, and in Samples 4 and 5 for the male, apparently at the expense of a lowered albumin in each case. That this component is lipoprotein in nature has already been suggested(13). Its appearance in both female and male sera, and at increased levels during periods of crop gland enlargement, indicates a possible relationship to the mechanism of pigeon milk formation, which takes place in both sexes of this species(1).

In addition to increases in protein and lipids, extraordinarily high levels of phosphorus and calcium have previously been found in avian plasma at time of ovulation (5,6,16,). Clegg and Hein(17) showed that in chickens, the increased (and non-diffusible) serum calcium bore no relationship to the albumin.



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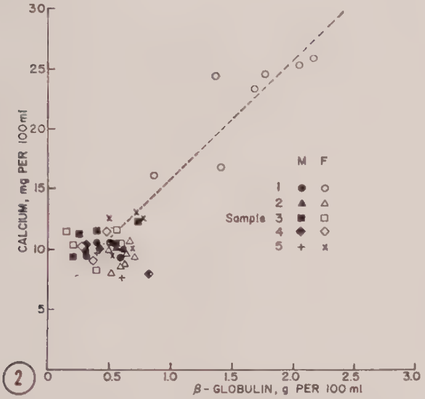

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FIG. 1. Electrophoretic patterns of sera of a pair of mated pigeons, at selected points (see text) in reproductive cycle. Ascending (a) and descending (d) patterns A, B, and C are shown for female Samples 1, 2, and 4, respectively; patterns D and E are for male Samples 1 and 4, respectively.

FIG. 2. Relationship of calcium to β-globulin concentration in serum of pigeons during reproductive cycle. Points represent individual analytical values. In units of concentration shown, $Ca = 10\ \beta\text{-Globulin} + 6$.

Clegg, *et al.*(15) related such extra calcium-binding ability under hormonal influence[1], to the presence of a fast moving high phosphorus-containing electrophoretic component. On addition of *extra* calcium to such serum, this leading component was found to migrate in the area of β-globulin. The authors concluded that the fast moving component operated *in vivo* as a globulin, and that the calcium-binding ability was the property of a lipoprotein.

In the 2 comparisons available(10,12), it has been shown that the electrophoretic characteristics of pigeon serum are markedly different from those of the chicken. Nevertheless, the present work on the former species is generally in accord with the observations and conclusions drawn from the foregoing, and other previous work on the domestic fowl, cited by McIndoe(18). Table II shows that as the reproductive cycle is followed, the serum calcium of female pigeons undergoes a marked increase, *pari passu,* with the increase of β-globulin at, and only at, the time of egg laying. Values for calcium, even more so than for β-globulin, are steadily maintained in serum at normal levels in the female of the other points of the cycle, and at all times in the male. In a statistical test of average serum calcium values of Table II, the datum for Sample 1 of the female was found highly significantly different $(P = <0.001)$ from the values for any other sample of either sex. The same was true for the β-globulin values. Apparently, no other serum component is involved in the increase of

TABLE II. Mean Concentrations of Total Calcium, Protein, and β-Globulin in Sera of Pigeons at Selected Points of Reproductive Cycle.

Sample No.	1		2		3		4		5	
Sex	F	M	F	M	F	M	F	M	F	M
					Total Calcium mg per 100 ml					
No. of Birds	7	8	8	7	5	5	5	6	6	7
Mean	22.6	9.9	9.4	8.5	10.4	10.9	9.7	10.6	11.7	9.2
S.D.	±4.2	± .6	± .9	±1.0	±1.3	±1.2	±1.3	±1.5	±1.5	±1.2
					β-Globulin, g per 100 ml					
No. of Birds	8	6	7	2	6	6	4	3	5	2
Mean*	1.59	.44	.60	.47	.35	.39	.39	.51	.64	.48
S.D.	± .41	± .11	± .07	± .13	± .19	± .22	± .09	± .28	± .12	± .17
					Total Protein, g per 100 ml					
No. of Birds	7	8	8	7	6	6	5	6	6	5
Mean	4.09	2.63	3.19	2.69	2.42	2.13	2.54	2.53	2.82	2.40

* Individual values obtained as product of individual total protein and β-globulin percentage values.

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[1] Electrophoretic patterns of the sera (in pH 8.6 borate buffer) of laying hens were found similar to those of diethylstilbestrol-treated cockerel(15).

cyclic physiologically mobilized serum calcium[2].

Roepke and Hughes(19) first suggested that the phosphoprotein in the serum of laying hens was related to the vitellin of the egg yolk. An accumulation of evidence in favor of this view has been cited by McIndoe(18) who recently isolated such a lipoprotein complex from the plasma of laying hens. It is therefore of interest, if only as a coincidence, that the line of variation of β-globulin concentration with *extra* calcium (above the normal concentration) in serum of laying pigeons (Fig. 2 for individual values) yields a result of 10 mg calcium per g of β-globulin, in comparison with a value of 8.3 mg calcium per g of the calcium-protein complex (vitellin) of laying chicken plasma, calculated by Winget and Smith(20) from *in vitro* experiments. From *in vivo* relationships for phosphoprotein bound -calcium and protein phosphorus (serum vitellin), established by McDonald and Riddle (6) for pigeons under a wide variety of hormonal changes, the value of 7.7 mg calcium bound per g protein (vitellin) is found. Obviously, many factors, some beyond experimental control, affect the results of a biological study of this kind. The frequency and extent of bleeding, diurnal(21) and seasonal(16) variations in plasma constituent concentrations, age(14,17), diet, and extent of confinement of the birds(22), pH of the samples(15), and the buffer used for electrophoresis(10), are a few of the variables which would make impractical a more serious attempt to calculate, from the present work, the extent of *in vivo* serum calcium-protein binding.

Inasmuch as no elevated serum calcium or β-globulin value has been observed for male pigeons at any time in these experiments, the finding of an increased value for either constituent in a normal (untreated) pigeon is clearly indicative of the female sex. It is suggested that from the practical standpoint, microchemical calcium analysis would be the most unequivocal, and paper electrophoresis possibly the simplest, way of detecting the sex of live pigeons, a matter which otherwise is apparently fraught with considerable difficulty(1).

Summary. At times of egg laying the serum of female pigeons showed a marked, significant increase in β-globulin and total calcium over the normal values found at all other times throughout the reproductive cycle, and at all times for the male. The relationship of calcium bound to β-globulin concentration was roughly in agreement with similar values calculable from previous work done by other methods. A fast moving component of mobility greater than that of albumin was present in many samples, male and female, particularly at times of milk formation.

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[2] In the present experiments, a new cycle was found to succeed the previous one within a span of 33 to 40 days. In 2 instances (when Sample 5 was taken at a time which subsequently proved to be too close to onset of a new cycle, a "spiky" β-globulin pattern was obtained for the female. The lipid content of these samples was too high for a meaningful measurement of the fractional areas of the electrophoretic patterns. These were the 2 samples (not included in the average for Sample 5) which showed the highest serum calcium (33.3 and 32.2 mg %) values.

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Occurrence and Titer of Isohemagglutinins in Secretions of the Human Uterine Cervix.*† (27022)

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Gershowitz, Behrman, and Neel(1) have demonstrated the presence of anti-A and anti-B agglutinins in the cervical secretions of normal women and suggested that this might play a role in the deficiency of type A offspring in incompatible marriages of blood type A fathers to type O mothers. An incompatible marriage is here defined as one in which the male possesses an ABO blood group antigen for which the female partner has the corresponding antibody. Behrman, et al.(2) found an excess of type O women as well as a greater than expected number of incompatible matings in a series of 102 childless couples studied, for whom no apparent cause for infertility could be found "by the most exhaustive gynecological and urological examinations possible," and who had been attempting to conceive for at least 5 years. Matsunaga(3) observed both a reduction in fertility and an almost 2-fold increase in the frequency of childless couples in an incompatibly-mated group compared with a compatibly-mated control group. These observations, together with the finding of antigenic dimorphism in human spermatozoa by Gullbring(4) and the demonstration of the presence of antibodies to sperm antigens in the serum of some women by Rao and Sadri (5), lend support to the hypothesis of an immunological selection mechanism operating at a preconception level.

The present study represents an exploration into the quantitative levels of ABO agglutinins in the cervical secretions of women representing the different ABO blood groups, the relationship of these titers to the day of the menstrual cycle, and to the level of agglutinins in the serum.

Materials and Methods. A total of 428 samples of cervical mucus was collected from 182 women. Three of these were postmenopausal while the remaining 179 were in the reproductive age group. Blood and saliva specimens were also obtained in most cases. These subjects were selected from several sources including the Outpatient Gynecology Clinic of University Hospital (largely patients with infertility problems), inpatients from the Ypsilanti State Psychiatric Hospital, volunteers from medical students' wives groups, student nurses, and patients attending the Ann Arbor Planned Parenthood Clinic. The representativeness of the first source may be questioned but in fact these individuals, who comprise about 60% of Group I, contribute about 50% of the positive cervical specimens in this group and thus do not appear to bias the sample.

The total of 182 women was divided into 2 groups—Group I, comprising 141 subjects, from whom only a single specimen of cervical mucus was obtained, and Group II, 41 subjects, from whom 2 or more specimens were taken at different times of the menstrual cycle.

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Essentially 2 methods of collection were employed. In each of a small number of volunteers a menstrual cup (Tassette) was inserted into the vagina at times other than during the menstrual flow, and left in place for about 4-6 hours while the subject continued her usual activities. After removal, the sample was obtained by aspiration of the mucoid material at the bottom or adhering to the sides of the cup. The second and most frequently used method of collection was the direct aspiration of cervical mucus from the cervical os by means of a specially devised polyethylene pipette attached to a 10 or 20 cc syringe. Storage time until analysis was variable.

Determination of antibody titer was by means of a standard doubling dilution titration technique in saline, using 2% washed red blood cells; the end point (the last tube showing agglutination) was expressed as $-1 \times \log_2$. The tests were incubated at room temperature for one hour and read macroscopically before and after centrifugation. All doubtful tests were reexamined microscopically.

Since viscosity of the mucus constituted a major technical problem, a neuraminidase-containing crude enzyme extract prepared from *Clostridium perfringens*‡ was added in very small quantities (0.01 to 0.02 ml to total volume of specimen). In a series of preliminary observations, this enzyme preparation seemed to have no effect on serum and saliva hemagglutinin titers even after more than 10 days of incubation at 4°C. Sufficient decrease in the mucilaginous consistency of the cervical secretions occurred within 24-48 hours at 4°C to permit performance of the test. Prevention of hemolysis of erythrocytes by the crude enzyme solution was accomplished by addition of 0.01 to 0.02 ml of a 1% solution of ethylenediamine tetraacetic acid (Versene) as a metal-chelating agent, but was not necessary when purified neuraminidase‡ was used.

The serum α and β isoagglutinin titers were determined in a similar way, testing against 2% suspensions of A_1, B, and O red cells, the O cells serving as a control. Determination of the secretor status was performed by an inhibition hemagglutination technique using boiled saliva diluted 1:20 with normal saline.

Results. The distribution of Group I and Group II subjects possessing isoagglutinins in their cervical mucus tabulated with respect to ABO blood types is shown in Table I. Agglutinins were present in 23.7% of Group I women, who were selected only because of their availability for this study. This ratio compares favorably with the 22.1% frequency of positives found in the earlier study by Gershowitz, Behrman, and Neel(1), using diluted specimens of cervical mucus. However, when multiple specimens were obtained from the same individual, viz. Group II, the proportion of individuals with at least one specimen positive for agglutinins rose to 63.4%. The number of samples obtained from women in Group II ranged from 2 to 15 with the median at 6 specimens.

The frequency of positive samples among all the women was found to be significantly greater in type O than in the combined sample of type A and type B women (X^2 with 1 d.f. = 11.56, $p<0.001$). Among those specimens with detectable antibody present, the mean anti-B titer *in the cervical secretions* was significantly higher in type O women than in type A women ($t' = 2.34$, $0.02<p<0.05$) and, correspondingly, the anti-A titer was significantly greater in type O women than in type B women ($t' = 2.61$, $0.01<p<0.02$).‡‡ However, such differences in mean antibody titer were not encountered when *serum* anti-B titers of types A and O women were compared, nor when *serum* anti-A titers of types B and O women were compared. This lack of difference held for both the cervical hemagglutinin positive group (for anti-B $t=1.75$, 46 d.f., $0.05<p<0.10$; for anti-A $t=1.39$, 37 d.f., $0.10<p<0.20$) and the entire series of 141 cases for which serum antibody titers were available (for anti-B $t=0.56$, 127 d.f., $0.40<p<0.60$; for anti-A $t=1.98$, 58 d.f., $0.05<p<0.10$).

The presence of anti-A and the presence of anti-B in the cervical secretions of type O women in Group I do not appear to be independent events ($X^2=17.68$, 2 d.f., $p<0.001$)

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‡ We are greatly indebted to Drs. Saul Roseman and George W. Jourdian of the Rackham Arthritis Research Unit, Univ. of Michigan, for supplying these materials.

‡‡ Cochran's approximation of the Behrens-Fisher test was used because the variances were distinctly different, $F(25,15)=4.87$ for anti-B and $F(27,6)=7.28$ for anti-A.

TABLE I. Cervical secretion hemagglutinins and blood type.

	Group I (single specimen)			Group II (multiple specimens)		
Blood type	Total No. tested	No. positive for cerv. hemaggl.	Mean titer ($\bar{x} \pm$ S.E.) —†	Total No. tested	No. with at least one specimen positive for cerv. hemaggl.	Mean titer ($\bar{x} \pm$ S.E.) —†
A	63	7	2.0 ± .436	19	9	1.46 ± .175
B	9	4	2.0 ± .408	6	3	1.0 ± .00
AB	9	0	—	0	—	—
O	54	anti-A only 8 anti-B only 6 both 7 Total 21	anti-A = 2.47 ± .585 anti-B = 2.31 ± .645	16	Both anti-A and anti-B = 7 anti-A only = 1 anti-B only = 0 **Both and anti-A only = 5 **Both and anti-B only = 1 Total 14	anti-A = 2.84 ± .227 anti-B = 2.36 ± .263
Totals	135*	32		41	26	

* Blood type not available in 6 additional cases.

† — Endpoint of doubling dilution method expressed as -1 x $\log_2$.

** In these 2 categories some specimens had both anti-A and anti-B while other specimens taken from the same subjects at other times had only anti-A or anti-B.

and in this respect these antibodies resemble the serum hemagglutinins. However, in contrast to the finding of both anti-A and anti-B in the sera of type O individuals, these antibodies are found either singly or together in the *cervical secretions* of type O women (see Table I).

As in the earlier study, no antibodies were demonstrable in the cervical secretions of the 9 AB women in this series. However, in 3 instances, all involving type A women, the cervical secretions appeared to contain agglutinins not present in the subject's serum and not consistent with her ABO blood group. Two of these subjects were A_2 and one A_1.

The secretor type was determined in 163 women in this study by testing samples of boiled saliva for presence of soluble A, B, and H specific substances. No difference in ratio of secretor to non-secretor types in the positive cervical antibody and the negative cervical antibody groups was found (see Table II).

Among 17 patients from whom 6 or more consecutive specimens of cervical mucus were obtained at various points in the menstrual cycle, no consistent pattern of fluctuation in titers was detectable.

TABLE II. Relationship of ability to secrete ABO substances in saliva to presence of hemagglutinins in cervical secretions.

	No. of secretors	No. of non-secretors	Total
No. of cervical hemagglutinin: Negative women	83	27	110
No. of cervical hemagglutinin: Positive women	41	12	53
Total	124	39	163

x^2 = .0053, 1 d.f.

Among women positive for cervical agglutinins, there is at most a weak correlation between serum and cervical secretion titers. Thus, the regression coefficients of cervical secretion antibody titer on serum antibody titer were −0.068 for anti-B agglutinin in type A women, 0.149 for anti-A agglutinin in type B women, 0.108 for anti-B agglutinin in type O women, and 0.430 for anti-A agglutinin in type O women. None of these regressions is significant, but the numbers involved are small.

Discussion. Two explanations for the occurrence of hemagglutinins in the cervical secretions are suggested by the results of recent work which are pertinent to the present study.

The first arises from the studies of the transplacental passage of immune anti-A and anti-B from mother to fetus. Rosenfield and Ohno(6) observed that in 90% of cord sera of group O infants of group O mothers both anti-A and anti-B agglutinins were present, but when the mother was type A or type B, only 35% of compatible infants demonstrated the presence of the appropriate antibody in their cord bloods. More recently, Freda and Carter (7) have demonstrated a selective transmission of isogglutinins across the placenta dependent upon the blood type of the mother, type O mothers transmitting larger amounts of antibody to the fetus than A_2 mothers and the placentas in A_2 mothers permitting a freer exchange than placentas of A_1 mothers.

These findings in addition to the preponderance of group O women found among mothers of infants with ABO hemolytic disease, provide strong clinical evidence that the isohemagglutinins produced by group O individuals differ from those produced by group A and group B persons. Biochemical evidence in support of this observation has recently been supplied by Rawson and Abelson(8), who found that type O individuals produce isoagglutinin composed largely of γ_2 globulins, of relatively low molecular weight, separating in the ultracentrifuge with the 7S component, and readily crossing the placenta. Non-O subjects, on the other hand, tend to produce increased amounts of 19S antibody located in the γ_1 fraction of globulins which are rarely found to cross the placental barrier. If these observations are confirmed, it would not be amiss to propose that a similar mechanism may be responsible for the selective appearance of antibodies of given molecular size in other body tissues and fluids such as those of the uterine cervix, if such antibodies are in fact derived from the blood serum.

The second type of explanation arises from the work of Straus(9) who has been able to demonstrate the occurrence of specific antibody in human vaginal mucus as well as serum in response to the local application of soluble typhoid antigen. If the existence of such a local antibody-forming mechanism can be substantiated for other antibodies, then a similar scheme might be envisioned for production of hemagglutinins in response to specific antigen, perhaps of bacterial origin, within the vagina. These same antigens may also be effective in distorting the measurable agglutinin titers in the cervical mucus by inhibiting their response both *in vitro* and, more importantly *in vivo* against sperm antigens. Whatever may be the origin of the cervical hemagglutinins, be it through passive transfer from the serum, local production, or both, they appear from the present studies to be related in a quantitative as well as qualitative way to the ABO blood group of the individual.

The foregoing serves to emphasize the possible complexity of the basis for the deficiency of type A and B children in ABO incompatible matings, especially of O women with A or B men, the evidence for which has recently been reviewed by Levene and Rosenfield(10). In view of the well-known occurrence of ABO hemolytic disease, most of the attention has centered on the action of maternal antibodies on the fetus as the basis for the deficiency (3,11,12). The present data strengthens our earlier suggestion of an alternative mechanism, involving selection at a gametic level. The relative role of each of these two mechanisms remains to be clarified.

Summary. An analysis of the cervical mucus from 182 women randomly selected, revealed that 23.7% contained isohemagglutinins when a single specimen was examined but when more than one specimen was obtained the percentage rose to 63.4%. Type O women had agglutinins in their cervical secretions more frequently than either type A or type B women, and, in general, the titers were higher. There was no significant correlation between cervical secretion titers and serum hemagglutinin levels. The occurrence of antibodies in the cervical mucus seemed to be unaffected by the secretor type. Observations on multiple samples obtained from 41 women failed to suggest any correlation between antibody titer and phase of the menstrual cycle. These findings extend the data which earlier suggested the hypothesis of ABO selection at the gametic level as a factor in explaining the deficiency of certain types of offspring in ABO incompatible matings.

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Ineffectiveness of Pitocin on the Sheep Gravid Uterus: The Role of Pitocinase.* (27023)

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Continuous slow infusion of a low concentration of oxytocin is highly effective in stimulating rhythmic contractility of the human uterus(1,2). With faster infusion, the uterus remains contracted. The effect of intravenous infusion on the uterus of other animals has not been tested although Assali *et al.*(3) have done so in a study on uterine blood flow in sheep. The present study was undertaken to compare the response of the myometrium of the ewe with that of the human to obtain a controlled means of stressing the fetus if the uteri of the 2 species behave similarly.

In pregnant women, rhythmic contractions of the gravid uterus can be induced in an otherwise virtually quiescent uterus. Strength and frequency vary progressively, within limits, with amount of pitocin infused(2). Sensitivity of the uterus increases as pregnancy advances. To obtain comparable outputs of contractility *(force, in mm Hg, X frequency of contraction per unit of time)*, Caldeyro-Barcia and Poseiro (2) found that Syntocinon dosage requirement decreased with increase in time of pregnancy.

We decided to investigate the sensitivity of the gravid sheep uterus in an exactly similar manner. Six pregnant ewes in the last half of gestation (circa 150 days) were used. Four ewes were lightly anesthetized with diallylethylbarbituric acid and urethane (Dial), 0.2 ml per kg of body weight, given intravenously. Two ewes were prepared under local anesthesia (Procaine) only.

The ewe was placed on her side and part of the uterus delivered through a midline abdominal incision. Intrauterine pressure was recorded by a polyethylene catheter admitted to the amniotic sac and connected to a pressure transducer. At the same time, umbilical artery and vein pressures were recorded by small catheters admitted through branches of these vessels, as the membranes were maintained intact and the uterus closed after the operation. The fetus was *in utero*, in its normal habitat (4). In addition, maternal respiration was recorded from an intrapleural trocar and carotid arterial pressure by a catheter in that artery; the trocar and catheter were connected to Statham pressure transducers. Records were obtained on an ink-writing Offner recorder.

Syntocinon, synthetic pitocin, was infused into the jugular vein of the ewe by a continuous infusion pump (Harvard). Dilutions and rates of infusion were carefully controlled. After an initial 20-30 minute recording period, infusion was commenced, always starting at the lowest infusion rate. The starting rate was either 0.76 or 1.9 $m\mu$ Syntocinon per min. After 20 min the rate of infusion was doubled. This was repeated successively until 191-1928 $m\mu$ per min was being infused.

In no case was rhythmic uterine contractility observed prior to, or during, infusion of pitocin. An increase in tonus (intrauterine pressure) occurred. It will be seen that the onset of increased tonus commences with dose levels of 0.76 and 19.1 $m\mu$ of Syntocin per min (Fig. 1). The lowest thresholds (0.76 and 1.9 $m\mu$ per

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TABLE I. Summary of Myometrial Response in the Ewe to Pitocin

Stage of Pregnancy (by fetal Kg wt)	Threshold of uterus to Pitocin (mμ/min)	Maximum tonus Developed (mm Hg)	Change in tonus over initial level (mm Hg)	Infusion rate of Syntocinon at max. tonus level (mμ/min)
0.6 (twins)	.76	10	10	10
1.25 "	1.9	10	10	382
3.0 (singlet)	7.6	(12)	(8)	(191)
3.75 "	7.6	15	14	191
3.75 (twins)	7.6	12	6	764
5.25 (singlet)	19.1	10	8	191

Figures in parentheses are below maximum level which was not determined.

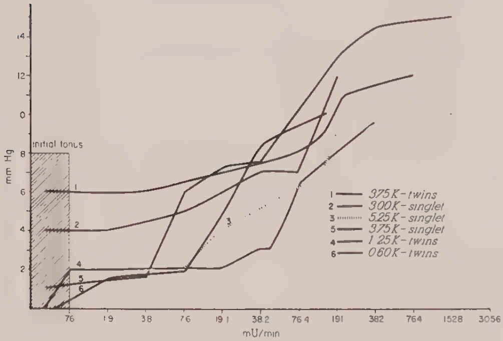

FIG. 1. Effect of pitocin on uterine tonus. Abscissa, amount of continuous infusion of pitocin per minute. Ordinate, pressure in mm Hg as recorded in the amniotic cavity.

min) are in the cases of the smallest fetuses (0.6 and 1.25 kg) in the middle trimester of pregnancy. The highest threshold (19.1 mμ per min) was in the case of the largest fetus (5.25 kg). The 3.0-3.75 fetuses were intermediate; the threshold for onset of increased tonus was 7.6 mμ per min.

Maximum tonus was only about 10-15 mm Hg, a fraction of the force developed by a mildly stimulated human uterus (40-60 mm Hg,3). The actual rise above the initial non-stimulated tonus was less (6-14 mm Hg), compared to the human (30-50 mm Hg). The infusion rate at the point of maximum tonus was very high (170-764 mμ per min) (Table 1).

No relation exists between rate of tonus increase or the magnitude of it and the initial, resting tonus, stage of pregnancy (i.e. fetal size) and the order of onset of increase of tonus with fetal size. There is no correlation between the half-way point of tonus increase and any of the foregoing factors. The data suggest that if initial tonus is high, the slope of the increase in tonus is low and conversely, that if initial tonus is low, the slope and height attained are high.

In short, the gravid myometrium of the ewe is relatively insensitive to infused pitocin, and it fails to exhibit the rhythmic contractility which the human shows under any reasonable dose of pitocin. The doses used span by many times the effective dose range used by Caldeyro-Barcia and Poseiro(2). Two post partum uteri and one non-gravid uterus of the ewe yielded results exactly comparable to the gravid uteri, as described.

Exploring for a basis for the difference between the ewe and the human, one is struck by a known but unappreciated fact. The human placenta is a source of the enzyme, pitocinase (5), and the blood levels of this substance reach a maximum at term when the human uterus is most sensitive to pitocin(6). On the contrary, the sheep is devoid of measurable blood pitocinase since only species having a hemochorial placenta elaborate this enzyme (7). We suggest that pitocin acts efficiently on the myometrium only in conjunction with the enzymatic splitting action of pitocinase. This interpretation accounts for the virtual non-reactivity of the uterus to pitocin in the ewe and the reactivity of the uterus observed in women. Moreover, it gives point to the otherwise unaccountable rise of a pitocin-inactivating substance at the time when, in women, pitocin is most effective.

Summary. Infusion of Syntocinon into pregnant ewes increased tonus but never induced rhythmic contraction of the gravid uterus. The infusion rates spanned a range of 0.76 to 1928 mμ per min. This is many times the effective infusion rate that elicits rhythmic uterine contractility in the human. Since sheep blood contains no pitocinase whereas that of the human

does, it is suggested that pitocin is effective in the presence of pitocinase.

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A Comparison of Some Properties of Polyoma Virus and Pneumonia Virus of Mice.* (27024)

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Polyoma virus (PY) and pneumonia virus of mice (PVM) are not related to each other serologically(1), but because of their similarity in size and their semi-ubiquitous presence in a latent form in mice, the possibility was considered that they belong to the same or related groups of viruses. Since sensitivity to ether serves to distinguish groups of viruses(2, 3), and since it is known that PY is ether resistant(4), the reaction of PVM towards ether was investigated. Ability of the 2 viruses to grow in tissue culture, and their *in vivo* effects on mouse lung were also compared.

Materials and Methods. Three strains of PY were used in these experiments. Strain LID-1 (5) was kindly provided by Dr. R. J. Huebner. Strain Lp or large plaque virus was derived by Dr. R. Dulbecco by single plaque isolation from a stock originally provided by Dr. W.P. Rowe(6,7). Sp or small plaque virus was isolated from transformed hamster embryo cells after exposure to Lp virus(7). Both Lp and Sp viruses were kindly provided by Dr. R. Dulbecco. The stocks of the various strains of polyoma assayed on secondary mouse embryo layers, according to the procedure of Dulbecco and Freeman(8), contained approximately the same number of plaque forming units, about 1×10^8 PFU/ml.

A single stock of PVM was used. This had been prepared by Dr. F.L. Horsfall, Jr. in 1953 and had been stored at −60°C since that time.

Standard procedures were used for intranasal infection of mice(9) and for preparation and infection of tissue cultures. Ether treatment was done according to the procedure of Andrewes and Horstmann(2).

Lung specimens were fixed in 10% formalin and stained with hematoxylin-eosin.

Results. Results of experiments on ether sensitivity of PVM are recorded in Table 1. Virus infectivity was measured by the ability to produce gross lesions in the lungs of weanling mice(9). The per cent of mice with lesions was plotted vs. dilution on probit paper and ID_{50}/ml was estimated graphically. In all experiments the virus appeared to be highly sen-

TABLE I. Ether Inactivation of Pneumonia Virus of Mice.

	Mouse ID50 per ml.	
Exp. No.	Control	Ether treated‡
1	7.5×10^4	No lesions at 10^{-2} dilution
2	5.8×10^4	idem. 10^{-1}
3	6.5×10^5	<7.0

‡ Virus was exposed to 20% ethyl ether for 18 hrs at 4°C.

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sitive to ether. In Exp. 3 large numbers of mice were inoculated with undiluted ether-treated virus. Lesions were produced in only a few of these mice, indicating that inactivation was almost complete.

The ether sensitivity of PVM suffices alone to eliminate this virus from the group containing PY. Nevertheless, several other properties of PVM were compared with those of PY. Various cell types were exposed to undiluted PVM and then observed for 6-14 days to determine whether PVM causes cytopathic changes in cells which are susceptible to PY. Three cell types were employed,—cell line L-929(10,11), primary mouse embryo cell cultures, and primary mouse kidney cell cultures. After monolayers had formed, the cells were washed twice and exposed for 60 minutes to 0.4 ml of a virus suspension containing approximately 1x10^5 mouse infectious units of PVM. After 2 further washings, nutrient medium was added to one set of plates and nutrient agar to the other set. Cells with nutrient agar were maintained up to 14 days and those with liquid medium up to 6 days. In no case was there evidence of a cytopathic effect.

Since PVM produces typical lesions in the lungs of weanling mice, the *in vivo* reaction of weanling mice to PY was tested. In a preliminary experiment a stock of LID-1 having a HA titer of 256 was used at a 1:5 dilution in phosphate buffer. Weanling mice were infected intranasally with 0.05 ml. After 9 days one set of 10 mice was sacrificed and their lungs inspected. Two mice had normal lungs, 6 had lungs of abnormal color, and 2 had extensive gross lesions. One month after infection the remaining mice were killed, serum prepared from the pooled blood, and the lungs removed and inspected. In this case 2 had normal lungs, 8 had extensive lesions, and 11 had smaller lesions and abnormal color. Representative lungs from both sets of mice were examined histologically and compared with mice exposed to PVM, to phosphate buffer alone, and to Pf broth containing 5% heated horse serum, the diluent for PVM. Lungs of mice exposed to phosphate buffer or to Pf broth were completely normal by both macroscopic and microscopic criteria.

Microscopically, the changes in the lungs of mice infected with PVM are typical of a viral pneumonitis with many scattered areas of edema of the bronchial, bronchiolar, and surrounding alveolar walls. An exudate filling the lumina of these structures consisted mostly of mononuclear cells, but also of a few polymorphonuclear cells and necrotic epithelial cells. In some areas there was an eosinophilic exudate with few cells, lining the alveolar walls. No hemorrhages were seen.

TABLE II. Effect of Dilution on Production of Lung Lesions by PY.

LID-1 was diluted in phosphate buffer. Weanling mice were infected intranasally with 0.05 ml per mouse. After 1½ months, mice were sacrificed and lungs were examined for lesions.

Dilution of virus	No. of PFU per .05 ml	Proportion of mice with lung lesions
1:5	1.8 x 10^6	14/27
1:50	1.8 x 10^5	5/8
1:500	1.8 x 10^4	0/8
1:5000	1.8 x 10^3	2/7
1:50,000	180	0/8
1:100,000	90	0/10

The lesions seen in the lungs of mice infected with PY were characterized by some congestion of the alveolar septa and large confluent areas of hemorrhage into bronchioles and surrounding alveoli. At times hemorrhages extended to the pleural surface. There was no evidence of collapse of alveoli nor was there evidence of an inflammatory response.

Hemorrhagic lesions were produced by LID-1 and Lp viruses, but Sp virus did not produce lesions, macroscopic or microscopic, even when used undiluted. The effect of dilution on number of lesions produced by LID-1 is shown in Table 2. Although it is clear that a moderately high dilution of virus results in formation of lesions, there are insufficient data to determine how many plaque forming units are needed to form a visible lesion.

The serum prepared from mice exposed to LID-1 did not have antibodies to PY. The HA inhibition titer of the serum was 1:64, which is not significant. Some lungs from mice exposed to 1:5 dilution of LID-1 were also harvested sterilely and homogenized as a 10% suspension in phosphate buffer containing 5% fetal calf serum. No infectious PY virus could be detected by plaque assay on monolayers of mouse embryo cells.

TABLE III. Comparison of Some Properties of Polyoma Virus and Pneumonia Virus of Mice.

1. Physical-chemical properties	PY	Reference	PVM	Reference
Size	45 $m\mu$	12, 14	40 $m\mu$	15
Relative heat sensitivity				
Stable at 60°C	Stable (30′)	13		
" " 56°C	"	13	Inactivated	16
Sensitivity to UV	Very insensitive	13	—	—
" " trypsin	Stable	13	Inactivated	1, 16
" " ether	"	4	"	*
2. Biological properties				
Immunological	Not related to PVM	1	Not related to PY	1
Hemagglutination	+	5	+	16
Lesions in mouse lung	Hemorrhagic	*	Pneumonitic	*
Cytopathic effect in mouse embryo cell cultures	CPE in 12-14 days	17	No CPE after 2 wks	*
Cytopathic effect in mouse kidney cell cultures	CPE in 4-5 days	18	No CPE after 2 wks	*

* Refers to this paper.

Discussion. A summary of comparative data on PY and PVM is presented in Table 3. All evidence clearly indicates that these viruses are unrelated to each other. The meaning of the hemorrhagic lesions produced by PY in mouse lung is not clear. The fact that mouse lung did not react to Sp virus may be in keeping with the generally lowered virulence observed with viruses of this type(19).

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Effect of Mouse Peritoneal Fluid on Strains of Enteric Bacilli.* (27025)

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The widespread occurrence throughout the subphylum Vertebrata of serum complement of very similar character suggests to us that the presence of this group of proteins may be of survival value to the animal species possessing it(1). One property of serum which may have survival value and is dependent upon complement is its ability to kill certain strains of gram-negative enteric bacilli *in vitro*(2). Evidence has been presented that enteric bacilli sensitive *in vitro* to the action of the complement-dependent bactericidal system of serum (i.e. serum-sensitive strains) are less likely than resistant strains to elicit bacteremia in man(3). Serum-sensitive strains do not survive as well as serum-resistant strains in the blood stream of the rabbit(4). Also it has been shown that serum-sensitive bacilli placed in intraperitoneal diffusion chambers are more likely to be killed by guinea pig peritoneal fluid than serum-resistant strains(5).

One serious objection to the proposal that the bactericidal activity of serum may be of survival value is the fact that, unlike man, the guinea pig, rabbit, and other vertebrates tested, the mouse does not possess a humoral bactericidal system which can be demonstrated by usual techniques *in vitro*(6). McGhee(7) was able to demonstrate *in vitro* a very small degree of hemolytic activity in normal mouse serum. Nevertheless several investigators(8,9) have confirmed the finding of Brown(10) that C′2 is missing in the mouse. Conclusions regarding C′3 and C′4 are not, however, in agreement. In the past, therefore, the lack of bactericidal activity of mouse serum has generally been attributed to an absence or relative deficiency of one or more of the major components of complement. Very recently a reëvaluation of this problem by Borsos and Cooper(11) indicated the presence of all 4 components of complement in mouse serum. They suggest that the lack of complement activity *in vitro* may be attributed to a serum substance which is perhaps actually one of the group of C′3 factors. Upon reaction of this substance with the C′1, 4, 2 complex a stable successor is formed.

In spite of this inability to demonstrate complement activity in mouse serum *in vitro*, the work of Carey *et al.*(12) indicates that complement may, nevertheless, be active *in vivo*. They reported that when gram-negative enteric bacilli are injected into the peritoneal cavity of the mouse, these bacilli undergo spheroplast formation and ultimate lysis. Under these circumstances complement may even play a rôle in determining the outcome of the host-parasite relationship. When an LD 50 inoculum of gram-negative enteric bacilli was injected into the peritoneal cavity, Carey *et al.* could, by determining the percentage of inoculated organisms undergoing spheroplast formation, predict whether the inoculum would be fatal.

Jeter and McKee(13) studied changes in resistance of mice to bacterial infections following intravenous injection of rabbit antiserum containing antibodies to mouse complement. Although they found decreased resistance to several gram-positive cocci, they found no discernible effect on resistance to gram-negative bacilli.

The Work of Rowley(14), while not discounting humoral factors, suggests that cellular activity plays the major rôle in removal of enteric bacilli from the mouse peritoneal cavity.

The present study was initiated to evaluate further the possible bactericidal property of mouse peritoneal fluid. In this study a modified Algire diffusion chamber provided an environment in which the fate of a bacterial inoculum could be determined when it was subjected *in vivo* to humoral defense mechanisms, yet remain relatively protected from cellular elements. It was of interest to compare, using the

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same test organisms, these results with those obtained in the guinea pig, which has an active complement system *in vitro*. The diffusion chamber also provided a means for the collection of peritoneal fluid for bactericidal studies done *in vitro*.

Materials and Methods. All seven organisms used were strains recovered from human cases of bacteremia or bacilluria with the exception of the 2 strains of *Salmonella typhimurium*.‡ These 2 typhimurium strains differ considerably in their virulence for the mouse. The less virulent strain (172) was derived from the more virulent strain (173) through passage on agar by Jensen(15). All mice used in these experiments were adult CF#1. The LD_{50} for CF#1 mice, when injected intraperitoneally, was between 9 and 90 organisms for typhimurium strain 173, and between 10^6 and 10^7 organisms for typhimurium strain 172. The sensitivities to active normal human and guinea pig sera were known for all the organisms used. In our experience, strains of bacteria sensitive to guinea pig serum have been sensitive in slightly varying degree to human serum; those strains resistant to killing by guinea pig serum have been resistant to human serum.

Sterile diffusion chambers were constructed from lengths of Tygon tubing, the ends of which were covered with Millipore membranes having a pore diameter of 0.45μ(5). These chambers have a capacity of between 0.3-0.5 ml.

To determine the permeability of the diffusion chambers to cells of the host we examined the fluid contents from the chambers for mammalian cells using phase microscopy. None were found. Upon removal of the chamber from the peritoneal cavity, the diffusion membranes were cut from the chamber after the host cells had been meticulously cleaned from the outer surface. These membranes were then stained with hematoxylin and eosin utilizing a procedure which rendered the membrane transparent. As demonstrated by others(16) a membrane with 0.45 μ average pore size is usually impermeable to cells of the host. Of 55 membranes examined at 24, 48, and 72 hours after the diffusion chamber had been implanted, five showed histologic evidence of host cell penetration. When host cells were present these were fewer than several hundred and these were located usually in a discrete cluster on the inner surface of the membrane. Chambers filled with inoculum showed no greater penetration by host cells than did chambers filled with saline. The percentage of membranes showing penetration by host cells did not increase when the chambers remained in the peritoneal cavity for several weeks. Fibrin clots removed from the diffusion chamber at termination of the experiment were fixed, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined for host cells. None were found.

Paper electrophoretic analysis of contents from chambers containing fluid in equilibrium with the peritoneal fluid showed a total protein concentration approximately 50% of that in normal mouse serum. The gamma globulin fraction in 5 samples of fluid removed from diffusion chambers averaged 13% of total proteins as compared to an average gamma globulin fraction of 9% for 6 samples of serum. Twenty-four hours after implantation of a diffusion chamber filled with 0.85% sodium chloride, total protein concentration was approximately one-third that in peritoneal fluid; 48 hr after implantation, total protein concentration was 85% of that in peritoneal fluid. Electrophoretic patterns of contents from diffusion chambers implanted filled with saline showed the appearance of some protein fractions at 8 hours. By 12 hr after implantation all major protein fractions of serum could be identified by paper strip electrophoresis. Fibrin clots appeared within the chambers filled with saline and occupied approximately 25%-50% of the volume of the chamber. No such clots were found in chambers which were implanted filled with air and allowed to fill with peritoneal fluid. The latter procedure was used to collect peritoneal fluid for the tests carried out *in vitro*.

A saline suspension of bacteria obtained from an 18 hour brain-heart broth culture was placed within the chamber before it was implanted in the peritoneal cavity. In further experiments an empty chamber was placed in the peritoneal cavity, and several weeks later, after the chamber had filled with peritoneal fluid, a small amount of this fluid was with-

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‡ The 2 strains of *Salmonella typhimurium* were obtained through the courtesy of Dr. D. Rowley.

TABLE I. Relationship of Sensitivity of Bacillary Strains to Bactericidal Effect of Normal Sera and Their Survival in Diffusion Chambers in the Guinea Pig and Mouse Peritoneum

Bacterial Strain	N Bacteria Killed/ml Serum in 2 hrs		Size of Inoculum	Growth in I.P. Chambers N Positive Cultures/N Trials	
	G. Pig	Mouse		G. Pig	Mouse
Klebsiella 15	160 x 10^6	0 (Growth)	<100	—	2/3
			100- 500	0/2	5/5
			1000-1500	0/5	—
Shigella 141	110 x 10^6	0 “	<100	—	0/3
			100- 500	0/3	5/5
			1000-1500	0/4	—
E. coli A9	170 x 10^6	0 “	<100	0/2	3/3
			100- 500	1/1	3/3
			1000-1500	0/4	—
E. coli 108	140	0 “	<100	—	5/5
			100- 500	—	3/3
			1000-1500	14/15	—
Sal. typhosa 202	400	0 “	<100	—	4/4
			100- 500	1/2	1/1
			1000-1500	1/1	—
Sal. typhimurium 172 (Sensitive)	95 x 10^2	0 “	<100		3/3
			100- 500	2/6*	3/3
			1000-1500		—
Sal. typhimurium 173 (Resistant)	650	0 “	<100	—	3/3
			100- 500	3/3	3/3
			1000-1500	—	—

* Growth or killing here was not related to size of inoculum. Organisms isolated from chambers in which growth occurred showed increased resistance *in vivo* to the bactericidal effect of guinea pig peritoneal fluid. This suggests that within the chamber in this situation a mutant more similar in sensitivity to the parent strain (173) may have been selected from the inoculum.

drawn under sterile conditions at laparotomy and replaced with an equal volume of saline containing a known number of bacteria. The fate of each bacterial inoculum was then determined at the end of the experiment by agar pour plates prepared from the chamber fluid. Most chamber experiments were terminated at 24, 48, or 72 hours.

The 5 enteric strains showing the greatest sensitivity to the bactericidal effect of normal human and guinea pig sera were used for experiments to determine the fate of such organisms *in vitro* in peritoneal fluid from the mouse. An inoculum of less than 150 organisms in 0.1 ml was added to 0.4 ml of peritoneal fluid. This was maintained at 37°C, and 0.1 ml aliquots were removed at intervals and agar pour plates made.

Results. Table 1 shows comparative results for survival of enteric bacilli in intraperitoneal diffusion chambers in the guinea pig and mouse. Note that of 47 experiments in the mouse the inoculum failed to multiply in only 4 experiments. In these 4 chambers the inoculum was either 30 or 35 organisms. The same strain grew when inoculated in greater numbers. It is possible that in the 4 chambers where growth failed to occur, the organisms failed to survive that interval of time in the saline before sufficient nutrients from the host had diffused into the chamber. Note also that size of inoculum was considerably smaller in the mouse than that used in the guinea pig. When the inoculum consisted of fewer than 75 organisms, the increase after 24 hours was in the order of 10^4 organisms. Regardless of size of inoculum, the increase after 48 or 72 hr within the chamber was in the order of 10^6 or 10^7 organisms.

To be certain that the time required for concentration of host proteins to reach effective levels within the chamber was not a critical factor in the fate of the inoculum, chambers already containing fluid in equilibrium with that of the peritoneum were inoculated as described under materials and methods. In such chambers inoculated with 32 organisms of *Salmonella typhimurium* strain 172, or 100 organisms of *Salmonella typhimurium* strain 173, growth at the end of 4 hours was almost 4 divisions per inoculated organism for strain 172, and about 4½ divisions per inoculated organism for strain 173.

Peritoneal fluid from the mouse failed to show bactericidal activity *in vitro* against 5 gram negative enteric bacilli tested. In most of these experiments unpooled samples of peritoneal fluid were used. No significant difference was noted between growth in normal peritoneal fluid which had been freshly collected, and that which had been heated at 56°C for 20 minutes. Growth in the peritoneal fluid was essentially as found in normal mouse serum which had been freshly collected, and in that which had been heated to destroy the activity of complement.

Discussion. Although it has been impossible to demonstrate bactericidal activity of mouse serum *in vitro,* Carey *et al.*(12) recently showed intraperitoneal formation of spheroplasts in the non-immunized mouse. This suggests that factors responsible for spheroplast formation, namely, natural antibody, complement, and perhaps lysozyme are active *in vivo.* It is possible, although unlikely, that peritoneal exudate of the mouse may possess properties not shared with serum. We have been unable to show *in vivo* or *in vitro* that peritoneal exudate of mouse possesses bactericidal properties. More likely, it may be that under the conditions in which Carey *et al.* demonstrated spheroplast formation, participation of peritoneal cells of the host was necessary. Our failure to find spheroplasts in diffusion chambers suggests this latter possibility.

For a given organism the correlation between the sensitivity to serum *in vitro* and survival within intraperitoneal diffusion chambers in the guinea pigs provides evidence that diffusion of humoral elements into this type of chamber occurs sufficiently quickly under these conditions to affect the fate of the inoculum (5). The techniques used in the mouse were essentially the same as those used in the guinea pig. In addition, in some of the mouse experiments an effort was made to demonstrate bactericidal activity by placing the bacterial inoculum into chambers containing fluid already in equilibrium with the peritoneal fluid, and further, by using a very small inoculum.

Rowley(14) has found that both virulent and avirulent strains of bacteria are killed by mouse peritoneal macrophages at similar rates. He feels that the limiting factor in the interaction of bacteria and monocytes is the rate of phagocytosis. Thus if *Salmonella typhimurium* strain 172 (avirulent) and *Salmonella typhimurium* strain 173 (virulent) are each placed in separate diffusion chambers, and rate of phagocytosis is the predominent determining factor as to outcome (i.e. virulence), then rate of growth within the chamber should be approximately the same for both these organisms. Our data confirm this. Additional support in favor of the importance of cellular mechanisms in resistance of the mouse to gram-negative enteric bacilli is the absence of killing in the diffusion chambers where all major serum protein fractions were present in concentrations comparable to those in the chambers in the guinea pig where killing did take place.

Further experiments are in progress to determine the effect of active immunization upon the fate of bacterial inocula in intraperitoneal diffusion chambers. Also, the fate of bacteria in the mouse is being determined after injection of the inoculum into diffusion chambers already containing mouse peritoneal macrophages.

Summary and Conclusions. Millipore diffusion chambers filled with gram-negative enteric bacilli and placed in the mouse peritoneum have been used to determine the fate of these bacilli in a cell-free environment. In contrast to findings in the guinea pig, all 7 strains of bacteria tested in the mouse grew in such diffusion chambers. These chambers also served to collect cell-free peritoneal fluid for bactericidal studies *in vitro.* Such studies showed mouse peritoneal fluid to have no bactericidal activity against gram-negative enteric bacilli.

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Antiproteolytic Power of Blood and Body Weight in the Albino Rat. (27026)

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The manifest antiprotease power (AP) of plasma as measured by its ability to inhibit tryptic digestion depends not only on the amount of antiproteases present but also on the amount of circulating proteases, on the physico-chemical state of the blood, and on the different affinities of the various inhibitor substances for trypsin.

This is a report on plasma AP of 143 albino rats (71 males and 72 females).

Method. 20 cmm of tail blood was withdrawn into a haemoglobin pipette and expelled into 10 ml of Sorensen's Phosphate Buffer pH 7.1. At the same time some blood was withdrawn into a heparinized capillary tube for determination of the packed cell volume (PCV). The blood-buffer mixture was immediately centrifuged for 15 minutes at 3,000 r.p.m. and the supernatant fluid used for antiprotease assay.

The assay was based on the determination of the optical density produced by the residual protein after incubation with protease, both with and without addition of plasma. The reaction mixture consisted of 1 mg Casein, 5 units Tryptar Armour (Lyophilized Crystalline Trypsin) and 0.001 ml plasma, made up to a total volume of 4 ml with Sorensen's Phosphate Buffer pH 7.1. The mixture was incubated for 15 minutes at 37.5°. The results are expressed in mg Casein saved from tryptic digestion by 1 ml plasma, this being the form of expression involving the least number of assumptions.

Results. Results are shown in Table 1. They were analysed from the point of view of sex, age, weight and PCV. Irrespective of sex, animals of the same weight show the same average plasma AP values. The PCV of these rats is low at birth and rises rapidly to reach a plateau of about 44% at the age of 8 weeks. The rising PCV correlates with the rising AP but thereafter the AP continues to rise while the PCV remains constant. It would therefore seem that concomitant rises during the first 8 weeks are not causally related. Plasma AP rises with age but the increase follows weight and not age.

When the weights of the animals regardless of sex, age or PCV are plotted against the AP, the data fit the quadratic equation: $AP = 252.7922 + 2.7083\ (G)\ - 0.004548\ (G)^2$. This is a statistically significant fit ($F = 10.4$; $n_1 = 1$, $n_2 = 136$; $P = 0.002$). Over the range of weights observed the data agree with the hypothesis that the change in antiproteolytic power is related to weight by a quadratic equation. It follows that there will also be a correlation with body surface.

Discussion. Plasma AP results from the actions of a number of inhibitor substances each having different affinities for at least 5 distinct proteases; (1,2,3,4,5). Changes in the plasma AP occur in response to a variety of distinct though not necessarily unrelated processes including absorption into the blood of pancreatic trypsin and antitrypsin, blood clot-

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* This investigation was carried out under the aegis of the N.Z. Medical Research Council. Mr. George F. Spears of the Otago Medical School did the statistical work and Miss Maureen Kennedy was technical assistant.

TABLE I.

MALES					FEMALES				
No.	PCV	Age	Wt G	AP Mg. Casein Ml. Plasma	No.	PCV	Age	Wt G	AP Mg. Casein Ml. Plasma
1	28	6 hr	3	222	1	27	1 hr	2½	233
2	26	24 “	4	270	2	29	6 “	3	240
3	22	24 “	5	205	* 3	142/4	12 “	20/4	309
4	22	24 “	5	205	4	32	3 days	5	294
* 5	116/4	12 “	20/4	364	* 5	162/5	6 “	30/5	328
6	26	3 days	5	337	6	32	10 “	7½	123
7	23	4 “	8	337	7	26	4 “	8	270
* 8	124/4	5 “	36/4	253	8	31	10 “	12	202
* 9	111/3	14 “	48/3	90	9	38	3 wk	12½	292
10	33	3 wk	16	298	*10	80/2	14 days	30/2	240
11	36	4 “	21	171	11	39	14 “	17	363
12	29	13 days	24	222	*12	90/3	13 “	63/3	324
13	29	3 wk	25	401	13	31	3 wk	25	145
14	31	4 “	34	318	14	37	7 “	37	158
15	46	8 “	38†	155	15	33	6¼ “	39	608
16	34	5 “	40	560	16	36	5 “	40	416
17	49	8 “	43†	686	17	34	6¾ “	45	530
18	35	6¼ “	45	171	18	36	6¼ “	45	312
19	34	6¾ “	54	515	19	33	7 “	48	350
20	47	7½ “	55†	362	20	36	5 “	60	434
21	34	5 “	57	364	21	39	7½ “	62	450
22	46	7½ “	69	444	22	49	7½ “	74	486
23	44	9½ “	79	339	23	39	8 “	80	818
24	47	7½ “	90	509	24	40	8 “	81	278
25	41	8¼ “	95	242	25	42	9½ “	81	362
26	41	10 “	117	379	26	42	8¼ “	83	522
27	44	7½ “	120	819	27	41	8¼ “	93	613
28	45	7½ “	120	627	28	38	7½ “	98	387
29	44	8 “	130	642	29	40	8½ “	105	690
30	42	10 “	130	443	30	43	8 “	111	544
31	43	8½ “	140	550	31	42	9½ “	112	534
32	42	9 “	145	379	32	40	9½ “	114	366
*33	138/3	12 “	486/3	659	33	43	8 “	120	724
34	43	14 “	171	400	34	45	8½ “	125	849
35	46	12 “	185	437	35	41	9 “	125	514
36	45	14 “	190	440	36	42	9 “	135	638
37	48	12 “	195	484	37	37	14 “	145	590
38	49	15 “	250	725	38	44	14 “	152	380
39	46	20 “	285	910	39	46	12 “	160	635
40	45	20 “	285	627	40	42	11½ “	160	562
41	44	22 “	285	500	41	48	12½ “	170	278
42	43	22 “	285	700	42	44	21 “	185	517
43	42	22½ “	289	825	43	44	32 “	185	535
44	46	22 “	290	481	44	47	19 “	187	641
45	47	20 “	290	685	45	44	32½ “	190	446
46	44	21 “	290	481	46	45	21½ “	190	690
47	48	21 “	290	576	47	44	21½ “	195	517
48	46	22½ “	290	611	48	43	32 “	200	280
49	45	22 “	290	672	49	41	25 “	201	610
50	44	20 “	293	696	50	42	34½ “	201	620
51	46	22½ “	293	870	51	44	39½ “	202	798
52	43	22 “	295	749	52	44	22 “	202	553
53	47	19 “	297	962	53	44	? “	210	500
54	45	21½ “	298	436	54	39	26 “	210	647
*55	90/2	22½ “	599/2	723	55	43	22½ “	210	482
56	49	20⅓ “	300	725	56	43	32½ “	210	582
57	46	21 “	300	740	57	45	39½ “	220	845
*58	92/2	23 “	610/2	437	58	45	34½ “	221	545
59	44	19 “	315	892	59	43	22 “	200	473
					60	41	22½ “	205	425
					61	43	22 “	210	491
					62	42	22½ “	216	474

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* Pooled sample (more than one animal).

† Starvation due to malocclusion.

ting, fibrinolysis, stress, and changes of adrenal cortical activity not related to stress (6,7,8,9,10,11,12,13,14,15).

Anaphylaxis also produces changes in plasma AP(7,16) and there are also species differences.

For these reasons the interpretation of changes in the manifest AP activity of plasma must be approached with great caution. Underlying the variables which have been mentioned there is a residual or basal AP activity which persists after pancreatectomy(10) and adrenalectomy(11,12). It is this basal AP activity in the plasma of normal rats which seems to be increasing with body weight.

Both in the rabbit and mouse mature animals have been stated to have higher plasma AP than immature ones(10,16) which is consistent with an increase in weight in these species. In our own laboratory, although we have so far found no significant difference in plasma AP of human beings from 2st.3lb to 13st.7lb in weight, average AP value of cord blood was found to be significantly lower(17). It must be remembered that the rat shows a 150-fold increase in body weight over a period of 6 months as compared with a 20-fold increase over a period of 25 years in the human. This may make the detection of biochemical correlates easier in the rat. Some of the inhibitor substances may have physiological actions which are not related to their ability to inhibit proteases, but their presence in the blood constitutes in fact an anabolic potential; as such it must have an influence on the protein and amino acid equilibrium of the blood. In view of the postulated existence of a dynamic equilibrium between circulating and somatic protein(18) plasma AP may therefore be related to total body metabolic activity.

Enzyme activity decreases with the B.M.R. for those enzymes which are directly or indirectly involved in the general metabolic processes(19). The mechanism of this decrease is not understood but a generalized increase in inhibitor activity could in part explain how a decreasing dissipation of heat leads to a decrease of metabolic rate. The rise in plasma AP with increasing body weight in the rat may simply reflect the falling B.M.R.

Summary. In the normal albino rat the antiproteolytic power of the plasma as measured by its ability to inhibit the tryptic digestion of Casein increases significantly with increasing body weight.

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Evaluation of Multichain Polyglutamic Acid as a Possible Plasma Expander.* (27027)

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The search for plasma substitutes is prompted by growing interest in their use in cases of simultaneous mass injuries. Many substances have been developed, but none fully conforms to the requirements of an ideal plasma volume expander(1,2). New substances are therefore being investigated continuously. The use of the natural α-glutamyl polypeptide isolated from *B. subtilis* as a possible plasma volume expander was examined by Bovarnick and his collaborators(3,4,5,6). When the molecular weight and diameter of the macromolecule were increased by coupling chemically several natural polyglutamic acid chains to yield a branched polymer, rate of renal excretion decreased considerably. Evaluation of sodium poly-α, L-glutamate as plasma expander has been reported by Kenny(7). At a concentration of 3%, sodium poly-α, L-glutamate was found to be similar to 6% dextran in its effect as a plasma expander, but the linear acidic polyamino acid at these high concentrations was too toxic for clinical trial.

The work reported here summarizes observations on the behavior *in vivo* of multichain polyglutamic acids(8), and their evaluation as plasma expanders. Multichain polyamino acids are synthetic branched polymers whose molecules are composed of linear polymeric chains attached to a polyfunctional core(9,10). The different polymer batches had polylysine cores of average degrees of polymerization of 70-140, and side chains varying in average length from 13 to 18 (Table I). The polymers are accordingly chemically described as multi-(copoly-L-glutamyl-L-aspartyl) poly-L-lysine.

Materials and Methods. The polymers were prepared by the method of Yaron and Berger (8). Three batches of different compositions and molecular weights were used (Table 1). Ultracentrifugation revealed that each batch contained about 15-20% of a low-molecular weight fraction.

The soluble sodium salts of the polymers were used throughout as 2-3% solutions in 0.9% sodium chloride solution. The solutions were adjusted to pH 7.0.

Seven mongrel dogs weighing 3.2 to 7.7 kg were fasted 12 to 20 hours before experiment, with free access to water. Six rabbits weighing 1.7 to 2.4 kg were used without preparation. The animals were anesthetized with intraperitoneal and intravenous sodium thiopentone. Hypovolemia was produced in some animals by arterial bleeding. Twenty to 30 ml/kg were removed in 10 to 20 minutes immediately prior to the polymer infusion. Other animals were given the polymer solution without prior bleeding. Twenty-five to 40 ml/kg of polymer solution was infused intravenously in 10 to 20 minutes. In 2 normovolemic rabbits the polymer dose was divided into 10

TABLE I. Amino Acid Contents, Molecular Weight and Sedimentation Constant of Polymers used in Infusion Experiments.

Polymer batch No.	No. of amino acid residues— in backbone Lysine	per one side chain Aspartic acid	Glutamic acid	Molecular Wt	Intrinsic sedimentation constant* (Svedberg units)
125/10	140	2.2	11.4	260,000	11.0
25/11	70	3.4	13.2	150,000	5.7
60/11	70	2.7	15	160,000	6.4

* Intrinsic sedimentation constant was determined on benzyl derivatives in dimethylformamide, 20°C.

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daily injections, no anesthetic being used. In one normovolemic dog the infusion lasted 1 hour. No anesthetic was added after starting the infusions.

Arterial blood pressure was measured in some dogs from the femoral artery with a mercury manometer and recorded on a kymograph. No anticoagulant was used. Blood hematocrit and erythrocyte sedimentation rate were measured on oxalated blood samples taken at intervals during experiments. Polymer concentrations in plasma and in urine were determined colorimetrically by the Safranin-O method of Bovarnick(4) with some modifications. Gross pathological examinations were carried out on animals that died.

Results. Plasma expanding properties. In all the animals that were bled prior to infusion, blood pressure dropped to shock levels after the bleeding. During infusion of the polymer solution blood pressure returned to almost normal levels. In all animals the infusion caused a drop of the hematocrit. The magnitude of this drop was that to be expected on the assumption that the polymer was initially distributed only in the plasma. No significant differences were found in these plasma expanding properties among the 3 batches of polymer, nor between polymer solutions of 2 and 3%.

Plasma and urine levels. During the first 2 to 3 hours after infusion, 10 to 20% of the injected polymer was recovered from the urine. Plasma polymer concentration dropped correspondingly by about 20% during the same period. No further polymer excretion in the urine was detected. The polymer recovered from the urine was shown by ultracentrifugation to represent the low-molecular weight fraction of the polymers. Subsequently plasma polymer concentrations dropped exponentially, reaching a level of 50% after approximately 48 hours (Fig. 1). In 2 rabbits the polymer dose was injected over a period of 10 days. Six days later the calculated total polymer in the plasma was 2.6 and 4.4% of the initial dose respectively. There was no significant difference in rates of disappearance from the blood among the 3 polymer batches.

Toxicity. The 2 rabbits which received the polymer dose in 10 daily injections showed no toxic reactions to the material. The other 4 rabbits showed increasing weakness and died within 21 hours. One rabbit, not bled previously, died with signs of pulmonary edema after 11 hours. All 7 dogs died within 6 days after infusion. During this period they suffered from diarrhea, loss of appetite, occasional vomiting and progressive weakness.

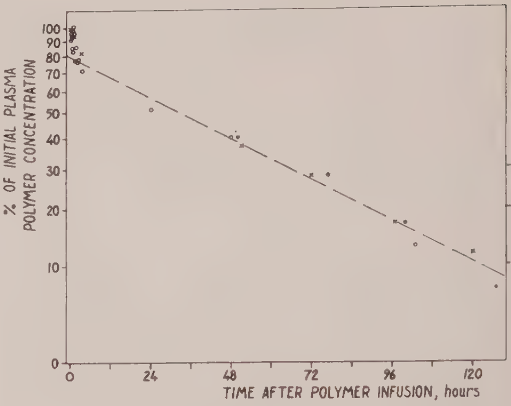

FIG. 1. Rate of disappearance of polymer from plasma in 3 dogs after intravenous infusion.

Apart from the restorative effect of the polymer solutions on blood pressure of the shocked animals, a transient fall of blood pressure occurred at the beginning of infusions in normovolemic as well as hypovolemic animals. A capillary bleeding tendency was observed at site of incisions. Blood clotting time was not prolonged. After death small quantities of bloody fluid were found in the serous cavities of some animals. Subserosal and submucosal hemorrhages were found in a few intestinal segments in most dogs and in one rabbit. Clumping of red blood cells and an increased sedimentation rate were caused by the polymer both *in vivo* and *in vitro*. Apart from these minor bleeding manifestations no macroscopic changes were observed after death.

Discussion. The polymer samples investigated were shown to be adequately retained in the circulation and were efficient in abolishing the features of hemorrhagic shock in rabbits and dogs, but they proved to be toxic. Severe gastrointestinal disturbances occurred, and mild hemorrhagic and hypotensive symptoms were found. Minor falls in blood pressure could be produced by rapidly injecting a few ml of polymer solution during infusion.

These toxic manifestations can be compared to the findings of Kenny(7), that linear synthetic polypeptides of glutamic acid caused a drop in arterial blood pressure in intact dogs, and that the dogs died within 5 days after constantly losing weight.

There is as yet no explanation as to the cause of toxicity of these polypeptides. The bleeding tendency did not cause major hemorrhages. The increased red blood cell aggregation and sedimentation rate are non-specific phenomena which are caused by all macromolecular substances which were investigated (11). The postmortem gross findings in our animals were not sufficient to explain their deaths.

The polypeptides investigated by Kenny, as well as our polymers, have multiple free negative charges whereby their oncotic efficiency is considerably enhanced due to the Donnan effect, as shown by Rosenthal *et al*(6). On the other hand, these negative charges may possibly interfere with the normal function of the cell membrane, and cause serious metabolic disturbances. It seems, therefore, desirable to extend this investigation to other, non-charged or isoelectric, water-soluble linear and multichain polyamino acids.

Summary. Some biological properties of 3 batches of a multichain polyamino acid, multi-(copoly-L-glutamyl-L-aspartyl) poly-L-lysine, were examined *in vivo* in dogs and in rabbits. The polymer was administered intravenously as 2 to 3% solutions of its sodium salt by one or by repeated injections. In some animals hypovolemia was produced by arterial bleeding prior to infusion. A low-molecular weight fraction of the polymer, amounting to about 10 to 20%, was excreted in the urine within a few hours. The rest of the injected polymer was not excreted in the urine, and disappeared gradually from the blood, with a constant half-concentration time of about 48 hours. As shown by its influence on blood pressure and on hematocrit, the polymer was able to combat the manifestations of hypovolemic shock. However, severe toxic manifestations appeared in many animals, consisting of diarrhea, vomiting, loss of appetite, a transient drop in blood pressure and a tendency to capillary bleeding. Progressive weakness of the animals was observed, leading to their death. The cause of toxicity of these polypeptides has not been found. Possibly their multiple negative charges may be responsible. It appears worthwhile to examine non-charged or isoelectric polyamino acids with regard to their plasma expanding properties and their toxicity.

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Preservation of Rabbit Spermatozoa: Fertility Results from Frozen Semen.* (27028)

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The influence of cold on animal spermatozoa has been studied for a considerable time. De-

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grees of cooling utilized in such early experiments have varied from $-4°C$ to $-269.5°C$ and living young have been produced from chickens, cattle, sheep and human beings from deep frozen ($-75°C$ or lower) semen(1-8). The recovery of one fertilized rabbit ovum has been reported by Smith and Polge(9) but no verifiable reports of young being produced from frozen semen have been made. Smirnov (10) stated that he produced young but gave no experimental data to support his assertions or to allow verification by other workers.

It is the purpose of this paper to report the production of 3 litters from does inseminated with frozen rabbit semen stored 24 hours at $-90°C$.

Materials and Methods. All semen samples were collected with an artificial vagina and the semen was deposited at the mouth of the cervices with an inseminating tube made of 6 mm glass tubing, 180 mm in length, with a 30° bend 60 mm from the insertion end.

The diluter was a slight modification of the CUE extender reported by Foote and Bratton(11), having the following composition: 20 cc egg yolk, 1.160 g sodium citrate ($NA_3C_6H_5O_7 \cdot 2H_2O$), .2400 g dextrose ($C_6H_{12}O_6$), .7496 g glycine ($NH_2CH_2COOH$), .1680 g sodium bicarbonate ($NaHCO_3$), .0320 g potassium chloride (KCl), 0.0100 g citric acid, 8.0 cc glycerine ($C_3H_8O_3$) and distilled H_2O to make a total volume of 100 cc.

Initial dilution of collected semen was at room temperature (20°C) using 0.15 cc raw semen per sample; the diluted semen was then cooled to 5°C during the course of one hour. Later dilutions, if made, were commenced 3 hours after collection.

The diluted semen was held at 5°C until freezing. Twenty minutes prior to freezing the diluted semen was transferred from rubber stoppered test tubes to 1/2 dram glass vials. Temperature of the vials was lowered during freezing by placing them into a $-25°C$ isopropyl alcohol and dry ice bath. Rate of cooling was controlled, by addition of chopped dry ice, so as to result in a rate of 5°C/minute to $-40°C$, 6°C/minute to $-52°C$ and 8°C/minute to $-70°C$. Samples were then placed directly in a mechanical freezer until thawing. Thawing was done in a 5°C water bath. After liquefaction, the vial was warmed in the palm of the hand for a few minutes and then further diluted just prior to insemination.

Litter 1. Semen was collected at 7:15 A.M. and 2 samples were diluted 1:10 with the complete diluter and placed in a refrigerator to start the cooling process. The semen was frozen 8 hours and 15 minutes later and stored at $-90°C$. After thawing, the 3.0 cc of diluted semen was further diluted by 1.5 cc of a sterile commercial bovine semen diluter (Nasco, Inc., Fort Atkinson, Wisc.) to stimulate motility, and then inseminated.

Litter 2. Semen was collected at 5:45 A.M. and 2 samples were used. One was diluted 1:10 with the complete diluter and the other was diluted 1:5 with a non-glycerolated diluter. Three hours and 45 minutes later an equal quantity of a glycerolated diluter containing 16% glycerine was added in the manner of Bratton *et al.*(12) except that a 15 minute interval was used instead of a 20 minute interval. Freezing commenced 5 hours after the first glycerine addition was made and the semen was stored frozen for 24 hours. To the 3.0 cc of thawed diluted semen, 1.5 cc of sterile diluter was added prior to insemination.

Litter 3. A semen collection was made at 6:55 A.M. and one sample (0.15 cc) was diluted 1:5 with the non-glycerolated diluter. Glycerol addition commenced 3 hours after collection in the same manner as in Litter 2 with freezing commencing 5 hours later. 1.5 cc sterile diluter was added to the 1.5 cc diluted, thawed semen prior to insemination.

Results and Discussion. A total of 6 does conceived out of 22 inseminations, based on palpation at 12 and 19 days. Three of these produced live young (litters 1, 2 and 3 with 3, 3 and 7 young born on May 14th, 16th and 22nd, 1961 respectively) and the remaining 3 pregnancies resulted in one doe resorbing at midpregnancy, and the other 2 each having one young which was carried *in utero* to 3 days over the normal 31 day gestation period, and then expelled as slightly resorbed fetuses. One of the 2 does which carried one young to 34 days was bred with semen stored 13 days at $-90°C$.

Ovulation was stimulated in all cases by use of a vasectomized male 15-30 minutes prior to insemination. All vasectomized males were checked by semen collections to insure complete vasectomy and litters 2 and 3 were positively identifiable based on color inheritance as being only from frozen semen.

Polge and Rowson(13) and Bratton *et al.*(12) have shown that storage time is not a critical factor for bovine semen preservation, thereby implying that the same may be true for the rabbit. Detailed experimentation is needed, however, to verify this for the rabbit.

Summary. Three live litters, totaling 13 rabbits, have been produced for the first time from frozen rabbit semen stored 24 hours at −90°C. The diluter used was a modified CUE diluter containing 8% glycerine. Vasectomized males were used to induce ovulation and genetic proof was presented to show that the young could not have come from the vasectomized males.

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Biosynthesis of Cholesterol XI. Biosynthesis of Cholesterol and Fatty Acids in Pregnant Rats.* (27029)

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The formal parallelism of the intrauterine growth of the embryo to tumor growth in the body of the host was the reason why the results obtained in this laboratory in a study of the biosynthesis of cholesterol and fatty acids in tumor bearing animals(1) suggested a similar study of the biosynthesis of cholesterol and fatty acids during pregnancy.

Methods. Virgin female Sprague-Dawley rats weighing about 270 g each were used. They were mated within 6 hours during the same day. All those animals which were allowed to carry to term gave birth to their young 21 days after mating. At the intervals given below for each experiment, a group of rats was injected with 0.5 mc of sodium acetate-1-C^{14} per kg body weight and 2 animals were killed 10, 20, 40, 80 and 240 minutes respectively after the injection. They were then dissected to obtain livers, gastrointestinal tracts, carcasses and fetuses. The tissues, after being weighed were hydrolyzed overnight on a steam bath in 30% KOH. After acidification, cholesterol and fatty acids were extracted with pentane. Purification of cholesterol and counting of samples were carried out as described before(2). Cholesterol determinations in serum were made with the method of Abell et al.(3) using the blood obtained by heart puncture from 4 animals for each determination at 6, 12, 20, 35 and 56 days after mating.

Experiments. Exp. 1: Ten non-mated rats were injected on the mating day with sodium acetate-1-C^{14} as described to serve as controls.

2: Ten pregnant rats were injected 6 days after mating. Because of the impossibility of separating the placenta or fetuses, whole uteri were used.

3: Nine pregnant rats were injected 12 days

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after mating. Because of the smallness of the fetus, placenta plus fetuses were isolated.

4: Ten pregnant rats were injected 20 days after mating. The fetuses were isolated.

4a: Five pregnant rats were injected 21 days after mating and not only the tissues of the mothers were isolated but also the fetuses were dissected and their livers, gastrointestinal tracts and carcasses collected separately. Three of the rats were killed 20 minutes and 2 80 minutes after injection of sodium acetate.

4b: Ten pregnant rats were injected 20 days after mating and not only the tissues of the mothers were isolated but also the fetuses were dissected and their livers, gastrointestinal tracts and carcasses collected. A series of 10 unmated rats were injected at the same time as the pregnant animals.

5: Ten rats were injected 22 days after mating (one day after parturition).

5a: Exp. 5 was repeated because of accidental loss of fatty acids.

6: Ten rats were injected 35 days after mating (14 days after parturition).

6a: Exp. 6 was repeated (using 9 rats) because of accidental loss of fatty acids.

7: Nine rats were injected 52 days after mating (31 days after parturition).

8: For this experiment especially purified cholesterol-H^3 with a specific count of 10 × 10^6 counts/min/mg was prepared(4). Five mg of it were dissolved in a little ether, mixed with 0.5 cm Tween-20 and the ether evaporated under nitrogen. This solution of cholesterol-H^3 was mixed thoroughly with 9.5 cm of serum from rats' blood, obtained by heart puncture. The serum mixture was incubated at 37°C for 4 hours and should contain cholesterol-H^3 bound as lipoprotein(5). Two-tenths cm of it was injected under slight pentobarbital anesthesia into the femoral vein of each of 10 pregnant rats on the 20th day of pregnancy. Two animals each were killed 10, 20, 40, 80 and 240 minutes after injection and livers, gastrointestinal tracts and fetuses isolated.

9: Cholesterol determinations in serum were made on the pooled blood obtained by heart puncture from 4 animals and taken at 6, 12, 20, 35 and 52 days after mating.

Results and Discussion. The results are presented in Fig. 1-5 and in Table I. Contrary to the findings in tumor bearing animals(1) they show that during pregnancy incorporation of radioactivity from acetate-1-C^{14} into liver cholesterol changes only slightly. This effect may be caused by the age difference between rats at beginning and at end of pregnancy since in experiment 4b control rats and pregnant rats show practically no difference in incorporation of radioactivity into cholesterol of the mother tissues (compare Fig. 3). However incorporation of C^{14} from acetate into cholesterol measured in counts per min per mg does not provide a complete picture. At the twentieth day of pregnancy (Exp. 4) total amount of cholesterol measured in mg (column 7 of Table I) shows a 50% increase over controls. Subsequently through parturition and lactation it falls off slowly to the original level. This increase in amount of liver cholesterol parallels the increase in liver weights in column 2 of Table I and is also in agreement with the serum cholesterol picture as shown in column 13 of Table I. Finally a value about 30% higher than in the normal animal is obtained. The higher serum figures persist through lactation and for 3 weeks after weaning, although at this time total liver cholesterol is at a normal level.

As soon as the young are born, counts in the liver cholesterol of the mothers (Fig. 1) increase considerably. This occurs concomitantly with milk production, and at the time when breast feeding is stopped cholesterol counts surpass by far the original values and those found at the beginning of lactation. Incorporation of C^{14} into the cholesterol of the embryo as shown in Fig. 2 seems to be of no particular significance in the earliest part of pregnancy whereas on the day before birth (Exp. 4) fetal cholesterol has a higher count than the liver cholesterol of the mother. Because the former is diluted with lower counting material from the carcass, Exp. 4a and 4b of Table I were made. Results of Exp. 4b are presented in Fig. 3. The fetal liver cholesterol contains up to 20 times more radioactivity than that from its own parent.

No impressive changes are seen in radioactivity of cholesterol isolated from the gastrointestinal tracts of the mothers during pregnancy and lactation. However the carcasses of

TABLE I. Cholesterol Biosynthesis in Pregnant Rats and in Fetuses.

Expt. No.		Total wt/ animal g 1	Liver wt g 2	G.I. tract wt g 3	Cholesterol mg/g			Cholesterol mg in livers of 10 animals 7	Total counts x 10-3 of cholesterol in			Yield§		Cholesterol mg in 100 ml serum 13
					Liver 4	G.I. tract 5	Carcass 6		livers 8	whole animals 9	in fetuses 10	in animals 11	in fetuses 12	
1	Control	266	10.2	22.5	2.17	1.37	.19	220	304	530	—	398	—	—
2	6 days*	241	9.1	18.9	2.18	1.47	.29	198	302	459	4†	390	3.6	42
3	12 "	281	13.1	27.9	1.92	1.27	.20	252	363	546	13‡	389	95.3	42
4	20 "	325	14.9	32.9	2.15	1.27	.20	320	264	454	540	279	332	78
4a	20 " Fetuses	335 2.6	13.7 .15	26.3 .1	2.24 2.06	1.16 1.78	— 1.69	306 —	— —	— —	— —	— —	— —	— —
4b	Control 20 days Fetuses	200 345 2.1	8.53 14.6 .19	23.2 34.1 .11	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— — —
5	1 day after part.	269	14.9	30.8	1.50	1.17	.62	223	332	536	—	398	—	54
5a	Repeat	286	13.0	33.4	1.65	.75	.31	215	324	491	—	343	—	—
6	14 days after part.	285	18.8	54.6	1.44	.99	.31	271	578	805	—	564	—	—
6a	Repeat**	288	16.0	49.5	1.69	.89	.62	270	778	1,051	—	729	—	—
7	31 days after part.**	257	11.9	27.0	1.94	1.04	.44	230	144	234	—	181	—	66

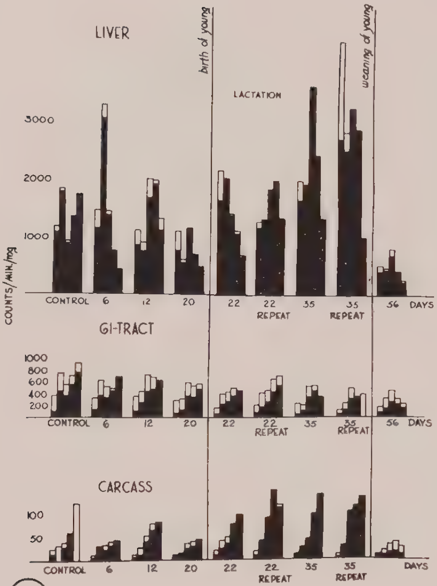
* Two animals may not have been pregnant.

** Only 9 rats survived, but figures are corrected for 10 animals.

† Uterus.

‡ Placenta plus fetus.

§ Yield $= \frac{10^2 \text{ x Column 9 (or 10)}}{.5 \text{ x Column 1}}$ Factor 0.5 derives from 0.5 mc per kg body wt of rats.



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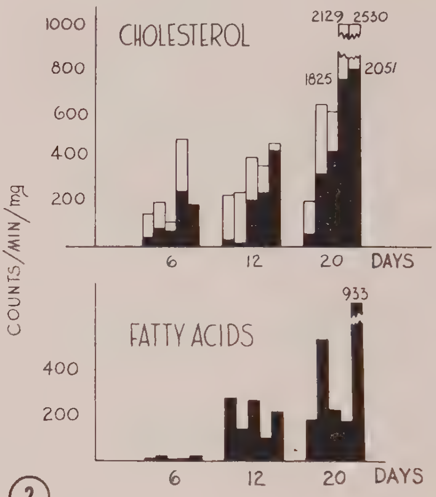

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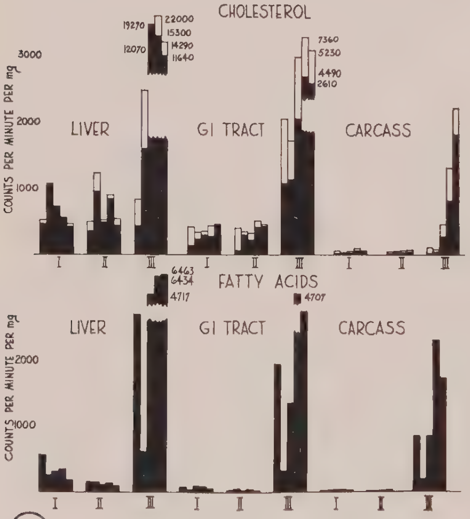

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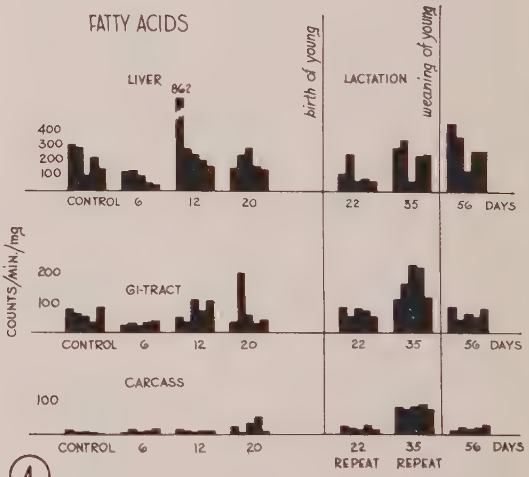

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FIG. 1. Exp. 1-7. Cholesterol-C^{14} counts/min/mg in tissues of rats. Each group of bars represents, as in the following charts, a series of rats killed 10, 20, 40, 80 and 240 min. after i.p. injections of sodium acetate-1-C^{14}. Total height of bar gives count in cholesterol from digitonide, black part of bar is count of cholesterol from dibromide.

FIG. 2. Exp. 1-7. Upper row; cholesterol-C^{14} counts/min/mg in uterus (after 6 days), placenta and fetus (after 12 days) and fetus (after 20 days). Lower row is counts/min/mg of corresponding fatty acids.

FIG. 3. Exp. 4b. Cholesterol-C^{14} and fatty acid counts/min/mg in tissues of mother rats and fetuses on 20th day of pregnancy. I are control animals. II are pregnant animals and III are the fetuses.

FIG.4. Exp. 1-7. Fatty acid counts/min/mg in tissues of pregnant rats.

lactating mothers (Fig. 1) contain cholesterol with higher counts than during pregnancy. Again on the day before birth gastrointestinal tract and the carcass cholesterol in the fetus (Fig. 3) has a much higher count than that in the mother's organs.

The counts of fatty acids (Fig. 4) in the organs of the mothers show an increase as the animals approach the end of pregnancy. This increase obtains during lactation and even a month after weaning of the young. In the fetus (Fig. 2 and Fig. 3) at the day before birth, radioactivity in the fatty acids is again very much higher than in the fatty acids of the mother.

During pregnancy there is a weight increase of livers (Column 2) and gastrointestinal tracts (Column 3 Table 1). This increase of 30% parallels the increase in total body weights. After birth of the young and during lactation weights of livers and gastrointestinal tracts continue to increase. At the end of lactation the percentage figure calculated on the now lower body weights are about 50% higher than at the beginning of pregnancy. It is interesting to note that the figures for the isolated cholesterol in milligrams per gram of wet tissue from livers and gastrointestinal tracts of the mothers (Column 4 and 5) remain nearly constant throughout pregnancy but then decrease considerably while the young are suckled and until they are weaned. The same figures calculated for the carcasses however (Column 6) increase after birth. All these changes regress after the young are weaned and one month after parturition the figures suggest a slow return to prepregnancy levels. The constancy during pregnancy of the figures for mg cholesterol per gram of wet tissue, suggests that the increases in liver and intestinal weights are not caused by an edema of these organs, which is confirmed by microscopic inspection. No increase in mitotic counts was observed.

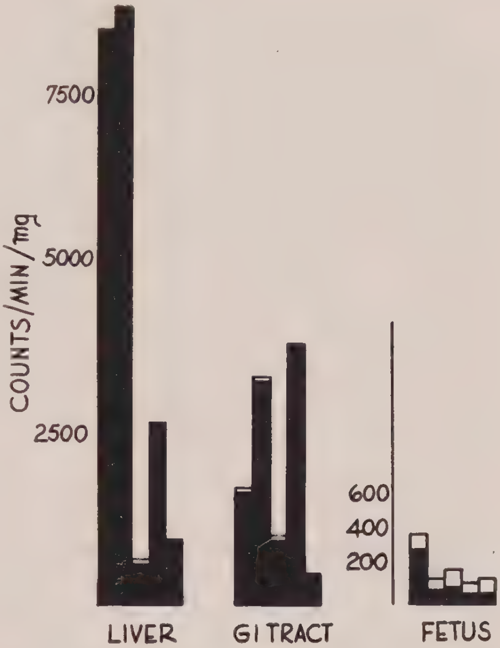

FIG. 5. Exp. 8. Cholestorol-H^3 counts/min/mg in liver and gastrointestinal tracts of pregnant rats and in fetuses on 20th day of pregnancy after intravenous injection of cholesterol-H^3 in rat serum.

It seemed surprising that incorporation of C^{14} from acetate into the cholesterol in fetal tissues appeared so much higher than in corresponding tissues of the mothers. Exp. 8 was therefore carried out to see whether cholesterol passes through the placenta from the mother and is merely stored in the tissues of the fetus. However, only an extremely small part of the radioactive cholesterol passes from the mother's blood into the fetus (Fig. 5). It is clear from this experiment that fetal cholesterol must be synthesized from a water soluble precursor like acetate or mevalonic acid transported by the blood stream through the placenta and then converted by the fetal liver. This raises the question whether the large difference between the activity of the liver cholesterol of mother and fetus could be caused by a higher efficiency in biosynthesis of cholesterol of the fetal liver or by the fact that the fetus does not oxidize cholesterol to bile acids, because it does not digest fat. Inspection of Fig. 3 (Exp. 4b) shows that incorporation of C^{14} into liver cholesterol in control as in pregnant animals occurs at a similar rate. Both values reach their peak at 20 minutes and by 240 minutes are down to 39 and 45% respectively of the peak value. In the fetus liver the highest counts appear somewhat later at 40 to 80 minutes and at 240 minutes are decreased only to 74% of the peak. Rate of decrease is evidently slower in the fetus than in the mothers. As each point in these charts represents the difference between biosynthesis and degradation of cholesterol up to this time, it is concluded that in the fetus degradation of cholesterol is slower than in the mother while rate of its biosynthesis may be similar for all 3 groups of animals. This difference between biosynthesis of cholesterol and its degradation is mirrored by the "higher counting companions"(2,6), the difference between the counts of digitonin-cholesterol and the counts of pure-cholesterol as represented by the white part of the bars in Fig. 3. They are much larger for the fetus tissues than for the mothers' and suggest a prevalence of biosynthesis over degradation in the fetus.

The highest figures for the counts in the cholesterol of the gastrointestinal tracts in controls, mothers and fetuses are observed at the end of experiment after 240 minutes. Rate of increase is nearly the same for the adult animals but about twice as fast in the fetuses. Figures for carcass cholesterol are again quite similar for control and pregnant rats but are considerably higher in the fetuses, where, at the end of the experiment, they reach 60 times the starting values.

The fact that the embryo in early pregnancy does not produce appreciable amounts of cholesterol is similar to our observation(1) that Yoshida ascites tumor cells do not synthesize cholesterol from acetate. In both instances the same mechanism may be active. The early cells of the embryo may likewise lack the ability to transform acetate into mevalonic acids, which as Popjak(7) has found is converted by tumor cells into cholesterol, while acetate is not. The fetal liver acquires the ability to use acetate only in the final period of pregnancy. The main part of radioactivity evidently passes via the blood stream from the mother into the fetus in the form of acetate because mevalonic acid could not be the precursor of the large amounts of fatty acids produced in the fetus at the end of pregnancy.

The questions of the relation between biosynthesis of lipids in mother and fetus have been studied before by Goldwater et al.(8) for rats and by Popjak(9) for rabbits. Both groups have come to the conclusion that if there is a passage of cholesterol from the mother into the fetus through the placenta, it can only be of minor importance and that cholesterol as well as fatty acids are mainly synthesized in the liver of the fetus. Popjak(10) also found that degradation of cholesterol and fatty acids in the fetus is lower than in the adult so that more of these substances can be accumulated in the tissues of the fetus. The findings here reported support the conclusions of these authors.

Summary. These investigations do not bear out the assumed similarity between the biosynthesis of cholesterol in pregnant rats with the same process in tumor bearing animals. During pregnancy there is apparently no appreciable impairment or other change in rate of biosynthesis in the mother liver although total amounts of cholesterol produced increase with the increase of weights of the livers during pregnancy and more considerably so during lactation, when milk production requires the synthesis of large amounts of fat. It is doubtful whether the early embryonal cell produces any cholesterol from acetate at all or accumulates it. However, at the end of pregnancy the liver of the fetus synthesizes this substance at least at a similar rate as the adult animal, in contrast to the observation that cells of the Yoshida ascites tumor do not make cholesterol from acetate. The degradation of cholesterol in the fetal liver at the day before birth seems to be much less than in the normal controls or the pregnant mothers, thus causing the apparently tremendous incorporation of C^{14} from acetate into fetal liver cholesterol. The experiment shows that only a very minute transfer of this substance from the mother through the placenta takes place. At the day before birth gastrointestinal tracts and carcasses of the fetuses accumulate much higher radioactivities than in the mothers. Synthesis of fatty acids increases during pregnancy in the mothers' tissues and reaches high levels during lactation. At the day before birth the tissues of the fetus show very high levels of radioactivity in the fatty acids.

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Carbohydrate Typing of Mycobacteria* (27030)

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Differentiation of mycobacteria species of medical interest has presented considerable difficulty due to a lack of definitive tests, especially with regard to the "atypical" or "anonymous" acid-fast organisms(1). Biochemical or metabolic testing should be preferred for precise differentiation of microorganisms, but slow-growing mycobacteria are classified at present primarily by cultural morphology and animal pathology. There are several useful biochemical tests, such as Konno's niacin test(2) and the succinamidase test(3), for distinguishing single species or strains of species. Other biochemical tests, e.g. the microcolonial neutral red and cording test(4), aid the grouping of mycobacteria. But no procedures have been reported which appear capable of aiding the typing of all kinds of mycobacteria. Organic acid utilization(5) offers some promise for general speciation, although no reactions were reported positive for the human tubercle bacillus and only one positive reaction was reported for *Mycobacterium avium*.

Carbohydrate utilization testing has not been applied generally to the mycobacteria due to technical difficulties. By adaptation of a procedure reported by Pickett and Nelson(6) for carbohydrate utilization of brucellae, differentiation of mycobacteria strains may be facilitated.

Materials and Methods. Organisms. Mycobacterial strains were inoculated from Lowenstein-Jensen egg medium (stock strains) into 50 ml amounts of TB Broth Base† with 0.5% sterile bovine albumin. No glycerol or other carbohydrate was added. Slow-growing organisms were incubated usually for 7 to 10 days, until just moderately turbid.

Preparation of Inoculum. The bacteria in 25 ml volumes of culture fluid were sedimented by centrifugation at 2,200 revolutions per minute for 3 to 5 minutes; clear supernates were poured off and discarded. Organisms sedimented from 25 ml of culture fluid were resuspended in 0.5 ml of basal medium without agar; 0.1 ml of the suspension was used as inoculum for a single carbohydrate test. The volume of packed wet cells per inoculum was 0.007-0.01 ml.

Basal Test Medium. Basal test medium for 100 tests was composed of: phenol red 4 mg, yeast extract 100 mg, K_2HPO_4 35 mg, agar 1 gm, distilled water 160 ml. The pH was adjusted to 7.0-7.2. Melted medium was dispensed in 1.6 ml quantities in screw-cap vials and autoclaved at 121°C for 15 minutes. Vials were cooled in vertical position; no slant was given the agar.

Carbohydrates. Carbohydrates were pre-

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TABLE I. Carbohydrate Reactions of Single Strains of Acid-Fast Microorganisms

	Slow Growers						Rapid Growers		
Carbohydrate	*M. tuberculosis*	BCG	Bovine Isolate	Swine Isolate	Scotochromogen	Photochromogen	*M. fortuitum*	*M. smegmatis*	*Nocardia asteroides*
Arabinose	–	–	–	–	–	W	–	+	W
Fructose	–	–	–	+	+	W	+	+	W
Galactose	–	–	–	–	–	–	–	+	+
Glucose	+	+	+	+	+	+	+	+	+
Mannose	–	W	+	+	+	–	+	+	–
Raffinose	–	–	–	–	–	–	–	+	–
Rhamnose	–	–	–	+	–	W	–	+	+
Sucrose	–	–	–	–	–	–	–	–	–
Trehalose	+	+	+	–	W	–	+	+	–
Xylose	–	W	–	+	W	+	–	+	–
Inositol	–	–	+	–	–	W	–	+	–

– = no acidification; + = strong acidification; W = weak acidification.

pared as 10% solutions, brought to a pH of 7.2, then sterilized by filtration. By overlayering 0.4 ml of carbohydrate solution on a 1.6 ml amount of agar base medium, final concentration of carbohydrate was 2%.

Testing Procedure. An inoculum of 0.1 ml of bacterial suspension was placed into the 0.4 ml of carbohydrate solution on top of the basal test medium. Vials were incubated at 37°C for 1 to 14 days, depending upon the strain of microorganism used and the particular carbohydrate. For prolonged incubation periods vial caps were tightened to prevent drying. Results were interpreted from change of the color of the phenol red indicator. Yellow (indicating acid production) was a positive result, red to purple was a negative, and orange was interpreted as either weakly positive or negative depending upon comparison with other carbohydrate tests with the same microorganism.

Results. Table 1 presents carbohydrate reactions by single strains of particular species or types of acid-fast microorganisms. Members of a fairly well-defined species such as *Mycobacterium tuberculosis* show similar but not always identical patterns. Strains of the same species generally resemble each other more closely in carbohydrate reactions than they resemble different species.

In the overall testing of various carbohydrates and polyhydric alcohols with many types of mycobacteria, glucose and glycerol usually gave strong positive reactions (acid production.) Weak positive reactions with other carbohydrates could, at times, be changed to stronger positive reactions by increasing the size of the inoclum, using younger organisms or modifying the medium. The acid-fast "yellow bacilli" have been reported to have generally low metabolic activities(7), and slow-growing pigmented mycobacteria often showed weak carbohydrate reactions.

Table II lists the results of testing 4 strains

TABLE II. Comparison of Carbohydrate Reactions of *M. avium* and Battey Strains

		M. avium					Battey			
Carbohydrate	Strain:	1	2	3	4	Strain:	1	2	3	4
Fructose		+	+	+	+		+	+	+	W
Glucose		+	+	+	+		+	+	+	+
Mannose		+	+	W	+		+	+	+	+
Ribose		+	+	+	+		+	+	+	+
Trehalose		–	–	–	–		+	+	+	+
Xylose		+	+	+	+		–	–	–	–
Adonitol		+	–	–	–		–	–	–	–
Glycerol		+	+	+	+		+	+	+	+
Inositol		–	–	–	–		–	+	–	–
Sorbitol		–	–	–	+		–	–	–	–

– = no acidification; + = acidification; W = weak acidification.

of *M. avium* and 4 strains of Battey type (non-photochromogen) organisms. These 2 types have been considered to be closely related by some workers and appear to be difficult to distinguish(8), although animal pathology clearly differentiates certain strains.

Discussion. Carbohydrate utilization has had extended use in general and medical microbiology for differentiating species or strains. Investigations of carbohydrate utilization by slow-growing mycobacteria have seldom been reported, and such investigations often produced uncertain results. The massive inoculum non-growth testing method employed in the present study provides the following advantages over earlier methods: 1) the test is sensitive to slight acid production because of the use of phenol red which changes color significantly around the neutral pH of 7, 2) nitrogenous constituents in the test medium are largely eliminated, thus preventing the masking of weak acidification by basic byproducts of nitrogen metabolism, 3) results can be obtained more rapidly than with growth tests, and 4) the test is read simply by noting the color of the indicator.

In the non-growth carbohydrate testing, large inocula are used. The bacilli are grown in a rich medium and are harvested in their logarithmic phase of growth which provides a high rate of metabolic activity. The cells in a dispersed condition present a large surface area capable of magnifying weak acidification to a point of simple visualization by means of a pH indicator. Earlier techniques depended upon the growth of microorganisms to show utilization of carbohydrate. Growth methods have the disadvantage that lack of growth might be due to several factors other than lack of carbohydrate utilization. A comparison of results from single strains of slow-growing and rapid-growing mycobacteria (Table I) indicates the potential usefulness of carbohydrate typing for separating species and types.

M. avium, Battey type non-photochromogens (isolated from human patients), and certain acid-fast bacilli isolated from swine resemble one another in colonial morphology and sometimes in animal pathogenicity. Phage typing distinguishes some of these strains(9), although the majority of such microorganisms are not susceptible to the phages available at present. Bojalil and Cerbon(10) studied carbohydrate utilization of *M. avium* and non-photochromogens (e.g., Battey types) using growth and the change of bromcresol purple indicator to represent positive reactions; only glucose and mannose of 18 carbohydrates tested gave positive reactions with some strains of each type of organism. Results reported here (Table II) suggest that *M. avium* and Battey strains may be differentiated by the carbohydrates trehalose and xylose in non-growth tests. Kubica and Beam(11) report that the arylsulfatase test readily distinguishes avian tubercle bacilli from Battey strains. Carbohydrate typing by the massive inoclum technique usually offers the advantage of yielding at least some positive test results, so that an organism is not differentiated by a negative test alone, as with the arylsulfatase test in which avian bacilli are negative. Although only a few strains of avian and Battey types have thus far been investigated with the non-growth carbohydrate procedure, all strains have given differentiable patterns of results.

Table III suggests that the trehalose and xylose reactions may be of further interest with regard to possible correlation to pathogenicity. Practically all proven and suspect human pathogens gave trehalose positive, xylose negative reactions.

The validity of the non-growth carbohydrate testing is indicated by the similarity of results

TABLE III. Potential Correlation of Carbohydrate Reactions and Pathogenicity.

Carbohydrate Reactions and Types of Mycobacteria	Probable Pathogenicity for Humans	Pathogenicity for Chickens
Trehalose +, Xylose − Human tubercle bacilli, some bovine isolates, Battey strains, *M. fortuitum* strains.	Pathogenic	Non-pathogenic
Trehalose −, Xylose + *M. avium*, some swine isolates.	Non-pathogenic	Pathogenic

obtained from both non-growth and growth techniques with rapid-growing mycobacteria species such as *M. smegmatis* and *M. fortuitum* which have had extensive previous carbohydrate investigation by growth techniques(12).

Some mycobacteria will surely be found to yield reactions intermediate to those of well-defined species. Also, some strains definitely belonging to a particular species may show individual reactions. Such "aberrant" or "peculiar" reactions should not occasion confusion; these reactions should lead to further study towards distinguishing organisms and towards correlating metabolism with various characteristics. Thus, special carbohydrate reaction differences between strains of the same species may correlate to differences in morphology, pathogenicity, and serology.

Summary. 1. A massive inoculum non-growth carbohydrate testing procedure provides results not obtained previously with slow-growing mycobacteria. 2. The results obtained indicate usefulness of carbohydrate typing for aiding differentiation of species and strains of acid-fast organisms. 3. The trehalose and xylose reactions may correlate with pathogenicity for certain types of mycobacteria.

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Modification of Solubility of Cholesterol During Incubation with Growing Cells of *Aerobacter aerogenes.** (27031)

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During the course of experiments involving the catabolism of C^{14}—labeled cholesterol by growing cells of *Aerobacter aerogenes*, a significant transfer of label into the acid fraction was noted. Subsequent studies indicated that this phenomenon was due, in large part, to production during cell growth of some factor which modified the solubility of cholesterol, rather than to a conversion of the sterol itself to an acidic product.

Methods. A strain of *A. aerogenes* described earlier(1) was grown on a sterile medium containing 1% agar, 1.5% Difco Nutrient Agar, 0.5% yeast extract, and 0.01% colloidal cholesterol(1) labelled with C^{14}, at 35°C for 2 to 4 weeks, either in screw cap bottles containing vials of strong alkali as CO_2 traps, or in cotton plugged flasks. Both randomly labelled cholesterol prepared by incubating

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1-C^{14}-acetate with rat liver slices(2) and purified via the dibromide(3), and 4-C^{14}-cholesterol (Nuclear-Chicago Corp.) were used. The purity of the latter compound was confirmed by paper chromatography using Bush's system A(4). At the end of the growth period, the mixture was hydrolyzed with 2N HCl by refluxing for one hour under N_2. Acid was used for hydrolysis, since the metabolized cholesterol was suspected of being linked to a carbohydrate by a glycosidic bond. The hydrolysate was made alkaline with $NaHCO_3$ and extracted 3 times with ether (separatory funnel). The aqueous phase was acidified with HCl in the cold, and again extracted in the same manner. The latter ether extract, termed acid fraction (AF), was then chromatographed on Whatman No. 1 filter paper. Either collidin/H_2O/NH_4OH (conc.) — 100:30:5 (v/v/v/) (5, with slight modifications), or Sjövall's system (mobile phase: isopropylether/heptane — 60:40 (v/v)) (6) were used as developing solvents. Following the development of the chromatogram, the paper was dried in an oven at 95-100°C, and then treated with either a saturated solution of $SbCl_3$ in $CHCl_3$, or a 2% solution of 2,4-dinitrophenylhydrazine in 2N HCl, for location of products.

Portions of the same chromatogram, untreated with color reagents, were cut into strips which were eluted with methanol. The eluates were placed on planchets, and after drying, their radioactivity was measured at infinite thinness using a gas flow counter.

Results. In the presence of growing cells of *A. aerogenes*, 6-10% of the total radioactivity of the cholesterol applied appeared in the AF (Table 1). The AF of identically treated, uninoculated controls contained no significant quantities of C^{14}. Nor did the AF prepared by a somewhat different procedure from resting cell samples, incubated with radioactive cholesterol for 7.5 hours, show detectable amounts of C^{14}(7).

TABLE I. Transfer of Radioactivity from C^{14}-Cholesterol to the Acid Fraction during Growth of *Aerobacter aerogenes*.

Exp.	Sample	Incubation time	Total radioactivity applied	Total radioactivity rec. in AF	
		weeks	cpm.	cpm.	%
1	Inoculated	2	23,500*	1,500	6.4
	"	3	12,400*	740	6.0
	Uninoc. control	3	12,500*	130	1.0
2	Inoculated	4	500,000*	47,100	9.4
	"	4	900,000	88,200	9.8
	Uninoc. control	5	500,000	6,000	1.2

* Avg of duplicate samples.

Paper chromatography indicated that 2 radioactive substances were present in the AF (Fig. 1, I). Spot *a* which contained about 98% of the radioactivity recovered from the AF, was identified as cholesterol by means of elution and rechromatography along with the known compound, using Bush's system A(4), and also by elution followed by digitonin precipitation, splitting of the complex(8), and rechromatography, using again Bush's system A (Fig. 1, II). The second radioactive spot, *b*, which contained about 2% of the counts, ran at a rate similar to that of cholic acid in collidin/H_2O/NH_4OH, but when rechromatographed in Sjövall's system(6), it traveled somewhat faster than the bile acid.

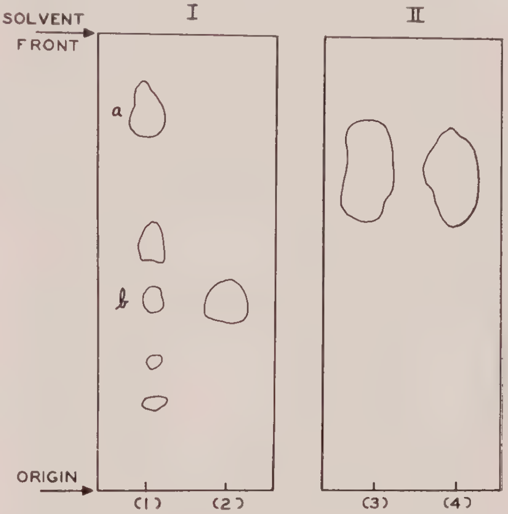

FIG. 1. I. Paper chromatogram of AF (1), and of pure cholic acid (2), developed with collidine/H_2O/NH_4OH. Only spots *a* and *b* contained detectable amounts of radioactivity. II. Paper chromatogram of radioactive compound eluted from spot *a* and purified as described in text (3), and of pure cholesterol (4), using Bush's system A. Staining with $SbCl_3$.

Discussion and Conclusion. It is seen that growth of *A. aerogenes* in a cholesterol-containing nutrient medium resulted in an alteration of the solubility behavior of the sterol: Significant amounts of the latter were found in the aqueous phase, apparently as the result of an artifact. Some substance, as yet unidentified, which was produced during cell growth, appears to be the responsible agent. The absence of significant quantities of C^{14} in the AF from both sterile incubated controls and incubated resting cell suspensions seems to preclude improper extraction, interfering substances in the medium used, or autooxidation during incubation and extraction as sources of this effect.

Increased water solubility of cholesterol in biological systems such as, for example, bile and serum(9, 10) has long been known. In addition, it is possible to modify the water solubility of this sterol *in vitro* by use of proteins, phospholipids(11), bile salts(12), or detergents(13).

It seems important, therefore, to use caution in interpretation of results of biological experiments dealing with cholesterol catabolism, in particular, where relatively small changes detectable only by means of tracers are involved. Simple classical extraction procedures, without further fractionation and a more detailed characterization of the products, were shown to be both inadequate and misleading.

Summary. C^{14}-labeled cholesterol was incubated with growing cells of *A. aerogenes.* Following hydrolysis of the incubation medium, a significant portion of the sterol was recovered in the saponifiable fraction. Apparently, the solubility of the sterol was modified by a substance produced during the growth of the *Aerobacter.*

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The Effect of Continuous Antibiotic Feeding on X-Irradiation Mortality in Mice. (27032)

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The occurrence of bacteremia in animals after exposure to lethal or sublethal total body X-irradiation has been reported(1-4). The bacteremia was caused primarily by microorganisms normally present in the lower intestinal tract; however, due to apparent increase in intestinal wall permeability these organisms were dislocated into the peritoneal cavity(1). Since the generalized infection develops during the period of greatest mortality, it may be concluded that infection plays an important role as one of the factors in death following irradiation. Various workers(5-6) have found that clinical doses of chlortetracycline when administered orally in the feed or by injection following irradiation prolonged the lives of rats, dogs and mice. The following experiments were undertaken to determine the effect of low level chlortetracycline feeding administered prior to and after exposure to total body X-irradiation on a lifetime basis. During the early weeks of the first experiment, a *Bartonella muris* infection was detected in the experimental animals. A second experiment was included to determine possible interactions of irradiation, disease, time and chlortetracycline feeding.

Methods. Mice. The mice used in Experiment 295 were young females obtained from Royal-Hart Animal Labs weighing 20-22 g. The mice used in Experiment 300 were young females of the Carworth Farms #1 strain weighing 19-21 g at time of irradiation. Initial group sizes varied with experiment and treatment (Table I).

TABLE I.
Experiment 295

Exp. No.	Group	Strain	No. Animals	Treatment Irradiation*	Chlortetracycline**
	2358	RH	116	–	–
295	2359	RH	111	–	+
	2360	RH	225	+	–
	2361	RH	217	+	+
		Experiment 300			
	2400	CF-1	100	–	–
	2401	CF-1	100	–	+
300	2402	CF-1	191	+	–
	2403	CF-1	191	+	+

* 250 Rad/Wk Total = 750 Rad.
** 20 ppm Chlortetracycline in Diet.

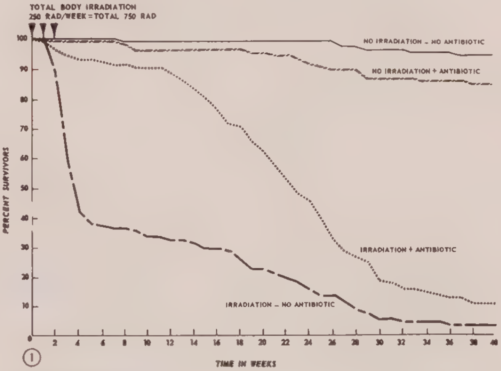
Diet. Ground Wayne rat pellets were fed to all mice upon delivery from the respective supplier for one week. Two weeks prior to X-irradiation diet 288R (Table II) was substituted as the basal diet and continued throughout the experiment. One experimental group and one control group in each experiment received supplements of 20 ppm (parts per million) chlortetracycline in the basal diet for duration of the experiment (Table I).

Method of Irradiation. All irradiated mice were exposed to 3 doses of 250 rad per week for 3 weeks for a total dose of 750 rad. Twenty-five to 30 mice were placed in a flat plastic cylindrical container, so that movement was limited to a minimum and only in a horizontal plane—during the exposure. The X-radiation was delivered from a 250 KVP X-ray unit at 30 ma. with 1.0 mm Cu + 1.0 mm Al filtration. Using an absorption value of 0.58 as an average quantity over the plastic cage and a transmission of 94% for the 1/8″ thick Acrylic Resin top, as determined by dosimetry measurements, the average dose rate was 51 rad per minute*.

Bartonella Detection. Blood samples were obtained from the tail and smears made. Staining of the organism was accomplished with

TABLE II.
Diet No. 288R Formulation

Material	%
Yellow Corn Meal	61.0
Soybean Meal (44% Protein)	20.0
Corn Gluten Meal	5.0
Fish Meal (Menhaden)	5.0
Alfalfa Meal	2.0
Distillers Solubles	2.5
Pulverized Calcite Limestone	2.0
Steamed Bone Meal	1.5
Sodium Chloride (Iodized)	.5
Vitamin Mix	.5
Vitamin Mix	
	Mg/Kg
Choline (25%)	206
Nopco (A + D dry)	206
Delamix + 2% Zinc	206
Crystalline B_{12}	2.3 (μg)
Riboflavin	.9
Niacinamide	.2
Ca Pantothenate	8.1



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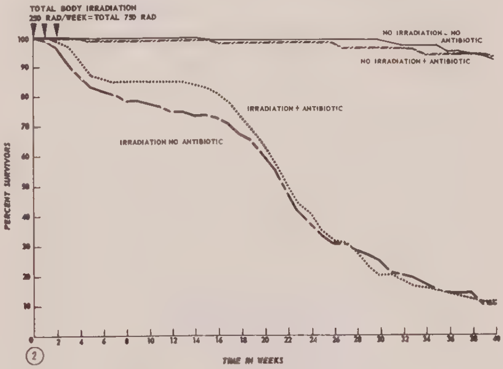

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FIGS. 1, 2. Effect of irradiation and chlortetracycline feeding on survival.

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* The authors wish to express thanks to Drs. R. F. Stamm and L. A. Siegal, American Cyanamid Co., Stamford, Conn. for dosimetry and irradiation service.

TABLE III.
Analysis of Variance

Source of Variance	Degrees Freedom	Variance
Overall Main Effects		
Radiation (R)	1	1606.88***
Chlortetracycline (C)	1	0.11
Disease (D)	1	10.58
Interaction		
(R) x (C)	1	12.50
Error	3	3.81
Least Required Variance	p = .05 = 38.60*	
	p = .01 = 130.00**	
	p = .001 = 638.18***	
Period x Treatment		
Main Effect		
Period (P)	3	257.10
(linear effect P′)	1	397.78**
(quadratic effect P″)	1	12.45
(cubic effect P‴)	1	361.08**
Interactions		
Period x Radiation		
(P′ x R)	1	339.54**
(P‴ x R)	1	210.13**
Period x Chlortetracycline		
(P′ x C)	1	31.90
(P″ x C)	1	174.85*
Period x Disease	3	47.73
Period x Chlortetracycline x Disease		
(P′ x C x D)	1	87.68
Period x Radiation x Chlortetracycline		
(P′ x R x C)	1	171.31*
(P″ x R x C)	1	177.85*
Error (Residue)	11	29.37
Least Required Variance	p = .05 = 142.14*	
	p = .01 = 283.42**	
	p = .001 = 578.30***	

10% alkaline Giemsa. Visual observation of the organism was used as positive identification of infection.

Results. The 40-week data are presented in Fig. I for Experiment 295 using mice with known *Bartonella muris* infection. One week following the final irradiation dose, a 60% mortality was observed. Following the initial high mortality, a decrease in rate was observed; however, mortality rate was in excess of that for the non-irradiated controls. Irradiated animals continuously fed 20 ppm chlortetracycline did not demonstrate the initial high mortality, although the overall mortality was in considerable excess of the controls.

Fig. 2 presents the total 40-week data for Experiment 300 with mice apparently free of *Bartonella muris* infection. The effects of irradiation are markedly different in the non-antibiotic group during the first 10 weeks compared with Experiment 295 since no initial acute mortality was observed. Under the conditions of this experiment there appeared to be little effect of the chlortetracycline on the increased mortality due to irradiation. Because of the obvious interactions involved, the data were statistically evaluated by the analysis of variance†. The statistical results are presented in Table III and Fig. 3. The effect of irradiation and period or time interval on survival indicated that mortality of the irradiated group is significantly greater during the entire period at the 0.001 level; however, it is interesting to note that the difference in mortality between irradiated and control animals is significant only during the first 30 weeks. The rate of mortality does not significantly differ during the final 10 weeks of the experiment. The overall and period effects of chlortetracycline without irradiation were without statistical significance. The effect of chlortetracycline on irradiation significantly modified the death rate ($p = 0.05$) during the first 20 weeks of the

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† The authors wish to express gratitude to Dr. R. H. White-Stevens for assistance with the statistical procedures.

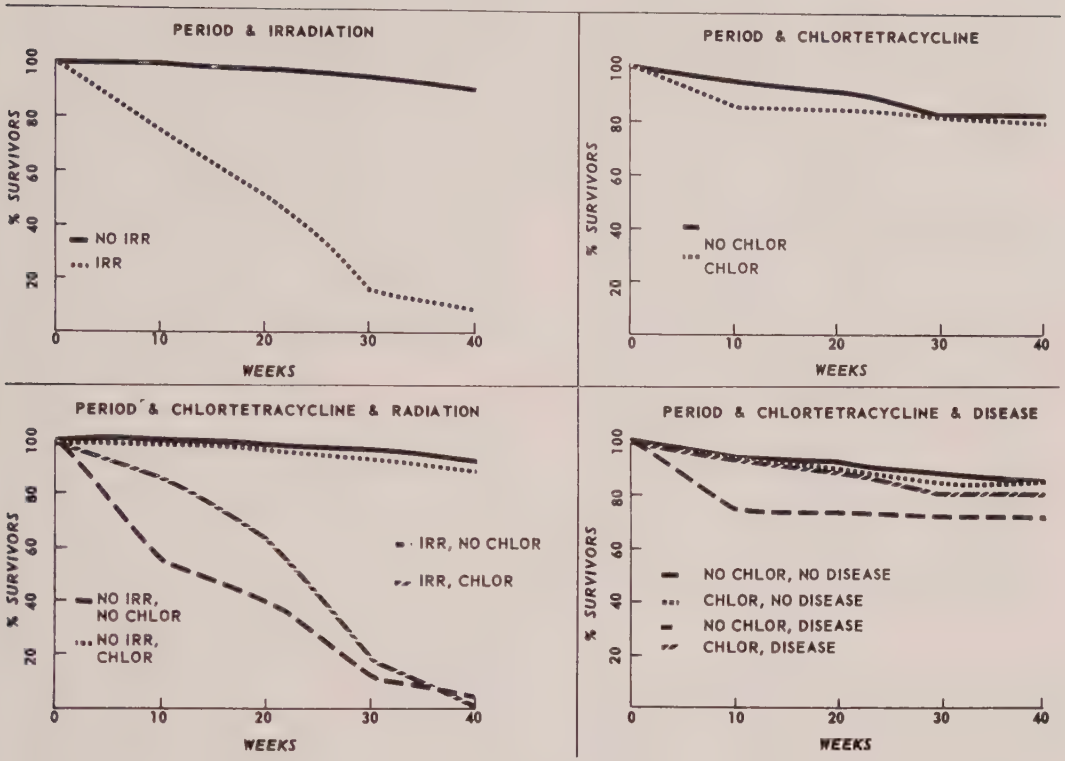

FIG. 3. Analysis of variance.

experiment; however, this effect was not significant during the latter one-half of the experimental period. The effect of the chlortetracycline was the result of a modification of disease during the first 10 weeks presumably due mainly to the effective control of *Bartonella muris* infection.

Discussion. The relationship of irradiation damage and bacteremia have been reported (1-3,7). Most of this literature deals with survivors of atomic bomb blasts or controlled irradiation exposure of relatively massive single doses with observations taken during the acute 30 day period following exposure. Other literature concerning the effects of antibiotics in conjunction with irradiation damage is primarily concerned with high doses of antibiotic and dislocation of intestinal bacteria. The original purpose of this experiment was to attempt to demonstrate a possible antibiotic effect on low-grade infections possibly due to irradiation damage of the immune mechanism. Unexpectedly, the acute mortality observed in Experiment 295, should not have occurred with this dosage procedure. *Bartonella muris* infection was suspected and established. The protective effect of antibiotic was due primarily to the susceptibility of this organism to chlortetracycline. The second experiment is also of interest because without the presence of the *Bartonella muris* infection, chlortetracycline did provide a protective or modifying effect on mortality during the early stages of the experiment, presumably due to antibacterial activity; however, overall effect of continuous chlortetracycline feeding was not statistically significant.

Summary. 1. X-Irradiation administered by the reported procedure significantly increased mortality over controls during a 40 week period. 2. Chlortetracycline administered in the feed at 20 parts per million significantly decreased irradiation mortality during the first 20 weeks of the experiment. 3. The marked chlortetracycline effect in Experiment 295 was due to modification of disease primarily due to *Bartonella muris* infection. 4. The lesser effect on mortality observed in Experiment 300 was probably due to antibacterial activity of the chlortetracycline on minor incident infections antagonized by the X-irradiation.

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Anticonvulsant Effect of Nialamide and Diphenylhydantoin.*‡ (27033)

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Evidence was accumulated during the past decade indicating that 5-hydroxytryptamine (5-HT), a normal constituent of the central nervous system, is of importance in the functioning of the system. Among its multiple functions, several investigators have suggested a possible interrelation between the levels of brain 5-HT and the excitability of the central nervous system. Prockop *et al.*(1) reported that the monoamine oxidase inhibitors have a pronounced anticonvulsant effect and that this effect is closely associated with an elevation of brain 5-HT and norepinephrine (NE). Reserpine which releases brain amines was reported to facilitate convulsions(2). Bonnycastle and co-workers(3,4) and Anderson *et al.*(5) have shown that a number of anticonvulsant compounds including diphenylhydantoin, mesantoin, trimethadione and paramethadione caused a significant elevation of brain 5-HT levels in rats and suggested a relationship between an increase in 5-HT in the brain and the anticonvulsant action of these drugs. Prockop *et al.* (1), on the other hand, observed that diphenylhydantoin does not increase amines in the brain of rats.

The purpose of this paper is to report the influence of pharmacological agents such as nialamide or reserpine which increase(6,7,8) or decrease, respectively, the levels of brain amines on the anticonvulsant action of diphenylhydantoin. A study of the interaction of diphenylhydantoin with these agents was of particular interest because nialamide has been reported to have anticonvulsive action(7,9) and reserpine to facilitate convulsions. Simultaneous measurement of brain amine levels under the various experimental conditions was also made.

Experimental. A total of 595 Swiss-Webster male white mice weighing 17 to 25 g and 125 Wistar male white rats weighing 100-120 g were used in these studies.

Two methods were used in testing the anticonvulsant action: 1. Protection against maximal electroshock seizures (MES) as described by Swinyard, Brown and Goodman(10) in which alternating current of 50 mA intensity in mice and 150 mA in rats with 0.2 second duration was delivered through corneal electrodes from a Hans Technical Associates Stimulator. The dose of anticonvulsant drug necessary to protect 50% of animals (ED_{50}) from the hindleg tonic extensor component of the maximal

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seizure pattern was determined. 2. Determination of the maximal electroshock seizure threshold, i.e., amount of current necessary to produce maximal tonic extensor seizures in 50% of animals (CD_{50}). All ED_{50}'s or CD_{50}'s and standard deviations were calculated by the use of Thompson & Weil's(11) tables for moving average interpolation. Unless otherwise stated, electroshocks were applied to the animals at 1, 4 and 5 hours after administration of diphenylhydantoin, nialamide and reserpine, respectively. No animal was shocked more than once.

To determine the influence of reserpine or nialamide on the anticonvulsant effect of diphenylhydantoin, groups of 20 to 30 mice were given a fixed dose of reserpine, 5 mg/kg I.P., or nialamide, 100 mg/kg I.P. Four hours later the animals were divided into groups of 5 each and a series of 4 to 6 geometrically spaced doses of diphenylhydantoin sodium was given to groups of the nialamide or reserpine pretreated animals. One hour after diphenylhydantoin, maximal electroshock (50 mA, 0.2 sec.) was applied, and the dose of diphenylhydantoin required to protect 50% of the nialamide or reserpine pretreated animals was determined.

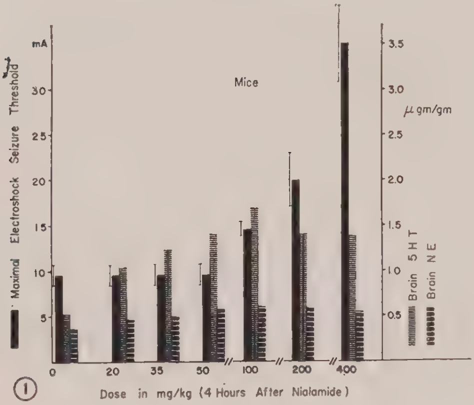
For determination of brain amines, the animals were sacrificed after application of electroshock and the concentrations of 5-HT and NE were determined spectrophotofluorometrically by the single extraction method of Mead and Finger(12).

Results. Effect of Nialamide on Maximal Electroshock Seizures and on Brain Amines. In mice, nialamide in doses up to 400 mg/kg had no protective action against maximal electroshock seizures using 50 mA current but it did increase the maximal electroshock seizure threshold. The convulsive threshold was unchanged after intraperitoneal doses of 20, 35 or 50 mg/kg of nialamide(Fig. 1). The levels of 5-HT in the brain, however, were twice that of controls after an intraperitoneal dose of 20 mg/kg and more than double the normal values after doses of 35 or 50 mg/kg of nialamide. A moderate increase in electroshock seizure threshold occurred only after the dose of nialamide had reached 100 mg/kg. It is interesting to note that in spite of the fact that brain levels of 5-HT had reached a plateau after 100 mg/kg of nialamide, there were further increases in electroshock seizure thresholds with increasing doses of nialamide. There was no significant change in brain NE content at all dose levels at the time the determinations were made.

Further evidence that the anticonvulsant effect of nialamide is independent of changes in brain amines may be found in the time course studies (Fig. 2). After intraperitoneal administration of nialamide, 200 mg/kg, there was a definite increase in brain 5-HT during the first hour and a marked increase during the second hour whereas an increase in electroshock seizure threshold was not observed until 4 hours after injection of nialamide. Sixteen hours after injection of nialamide when the brain 5-HT was still elevated, the antielectroshock action had disappeared and the electroshock seizure threshold had returned to normal. No consistent change in NE level was observed.

In rats, nialamide also increased the maximal electroshock seizure threshold and this effect again appeared unrelated to changes in brain amine levels. After intraperitoneal injection of nialamide, 35 mg/kg, the brain 5-HT reached its peak at about 4 hours and remained elevated for at least 24 hours (Fig. 3). The maximum elevation of seizure threshold was obtained 8 hours after nialamide. Twenty-four hours after injection of nialamide, when the brain amine levels were still as high as at 4 hours after the drug, the seizure threshold had almost returned to predrug levels. NE levels were practically unchanged.

Effect of Nialamide or Reserpine on Brain Amine Levels and on Anticonvulsant Activity of Diphenylhydantoin. The influence of nialamide and reserpine on the antielectroshock seizure effect (MES) of diphenylhydantoin in mice is shown in Fig. 4. When given alone, diphenylhydantoin (6.6 mg/kg) protected 50% of the mice (ED_{50}) from maximal electroshock seizures due to 50 mA intensity. The potent anticonvulsant action of diphenylhydantoin in mice was not associated with any measurable change in brain amines. Diphenylhydantoin in dose of 10 mg/kg protected 100% of the mice from MES, but even doses as high as 55 mg/kg did not cause any change in level of brain amines. When mice were pretreated



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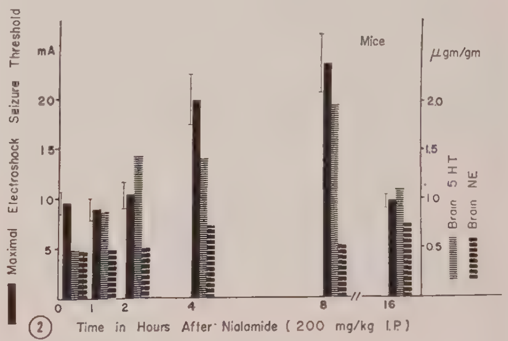

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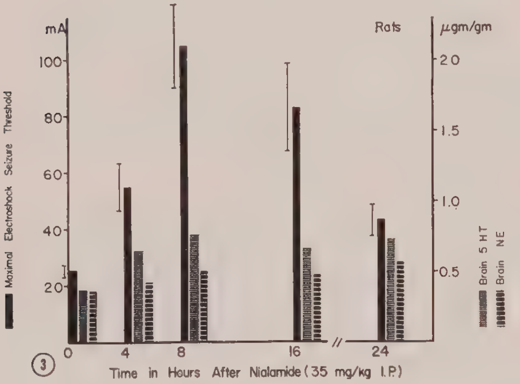

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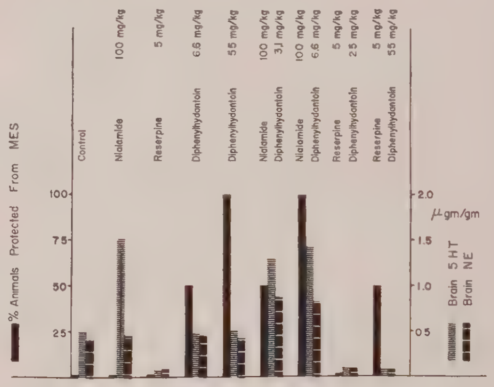

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FIG. 1. Relationship between increase in maximal electroshock seizure threshold and brain 5-HT and NE after intraperitoneal injection of increasing doses of nialamide in mice. Vertical brackets represent ±1 standard deviation.

FIG. 2. Relationship between increase in maximal electroshock seizure threshold and brain 5-HT and NE at different times after a single dose of nialamide in mice. Vertical brackets represent ±1 standard deviation.

FIG. 3. Relationship between increase in maximal electroshock seizure threshold and brain 5-HT and NE at different times after a single dose of nialamide in rats. Vertical brackets represent ±1 standard deviation.

FIG. 4. Interaction of nialamide or reserpine with diphenylhydantoin on maximal electroshock seizures and brain amine levels in mice. Nialamide or reserpine was given 4 hours before diphenylhydantoin. All drugs were injected intraperitoneally.

4 hours previously with 100 mg/kg of nialamide, the anticonvulsant effect of diphenylhydantoin was greatly potentiated, the ED_{50} was reduced to 3.1 mg/kg, i.e., one half of the dose required when administered alone. Administration of diphenylhydantoin to nialamide pretreated animals did not further increase the levels of 5-HT in the brain from levels of those that received nialamide alone. Levels of NE were, however, increased. Pretreatment of mice with reserpine 4 hours prior to administration of diphenylhydantoin markedly decreased the anticonvulsive effects of diphenylhydantoin; the ED_{50} for diphenylhydantoin in these reserpine pretreated animals was 55 mg/kg, i.e., almost 9 times the dose required when given alone. Administration of high doses of diphenylhydantoin to the reser-

pine pretreated mice did not reverse the depletion of brain amine levels elicited by reserpine.

Discussion. The elevation of the convulsive threshold produced by administration of nialamide to mice and rats does not appear to be directly related to changes in brain serotonin or norepinephrine for the following reasons: 1. Doses of nialamide which more than doubled the brain serotonin level did not increase the convulsive threshold in mice. 2. The brain serotonin concentration was not further elevated by doses of nialamide in excess of 100 mg/kg while the convulsive threshold continued to rise as the dose was increased. 3. A 2- to 3-fold elevation of brain serotonin was achieved at a time (2 and 16 hours) when no significant changes in convulsive threshold were observed. 4. Changes in convulsive threshold occurred without corresponding changes in brain norepinephrine. These results indicate that the changes in convulsive threshold obtained after nialamide are due to a mechanism of action other than elevation of brain serotonin and norepinephrine.

Diphenylhydantoin in doses from 6.6 to 55 mg/kg, which was almost 9 times the anticonvulsive dose, did not change 5-HT or NE levels in the brain of mice. Prior administration of nialamide potentiated the anticonvulsant activity of diphenylhydantoin but the levels of brain 5-HT were not higher than when nialamide was given alone. The concentration of brain NE was elevated by the combination of the two drugs. However, since it was shown that the anticonvulsant effect of nialamide or diphenylhydantoin was independent of changes in brain NE concentrations it does not seem likely that nialamide potentiated the effect of diphenylhydantoin by increasing the level of NE. Administration of reserpine facilitated tonic extensor seizures and while diphenylhydantoin was capable of counteracting this effect, very large doses of the anticonvulsant drug were required. This blockade of reserpine-induced facilitation, however, was not accomplished by reversal of the effects of reserpine on the brain amines. The failure of diphenylhydantoin to reverse the amine depleting effects of reserpine appears to corroborate the report of Chen and Bohner(13) who found that diphenylhydantoin did not reverse reserpine-induced ptosis, a response attributed by Costa and Pscheidt(14) to depletion of brain serotonin.

The results of this study demonstrated that while the elevation of the seizure threshold elicited by nialamide was not related to changes in brain amines, the possibility remains that a modifying action of nialamide or reserpine on the anticonvulsant action of diphenylhydantoin could be due to changes in brain serotonin and norepinephrine.

Summary. 1. Nialamide increased the maximal electroshock seizure threshold in mice and rats. This effect was independent of the elevation of brain 5-HT and NE elicited by nialamide. 2. Diphenylhydantoin in doses from 6 to 55 mg/kg, which was almost 9 times the anticonvulsive dose, did not significantly alter the content of 5-HT or NE in the brain of mice. 3. Nialamide potentiated and reserpine decreased the anticonvulsant effect of diphenylhydantoin. No correlation could be found between these effects and brain amine levels.

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Effects of Digitoxin on Exchangeable and Tissue Contents of Magnesium.* (27034)

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While the effects of digitalis on the myocardium are well known, its extracardiac effects are not. The similarity in chemical structure between digitalis and other steroid compounds known to alter salt and water metabolism would suggest that they might have similar effects. It has been shown, in fact, that under certain experimental conditions digitalis does act in a fashion similar to the adrenal cortical hormones(1,2). In a previous study on rabbits, administration of large doses of digitalis produced a significant reduction in the exchangeable body content of potassium and in the potassium content of many tissues(3).

It has not been known whether or not digitalis induces any alteration in metabolism of another important intracellular cation, magnesium. Since the availability of a radioactive isotope of magnesium, Mg^{28}, has made possible an exploration of this problem, the present experiment was undertaken to determine the effects of digitoxin on magnesium metabolism in the rabbit. Mg^{28} was used to measure the exchangeable body content of magnesium and its relative tissue uptake.

Material and methods. Twelve normal, male young adult rabbits were placed in individual stainless steel metabolism cages and fed a stock diet of compressed pellets. Tap water was given without restriction.

Commercial crystalline digitoxin dissolved in a 40% solution of alcohol was employed.

Mg^{28} was received as $MgCl_2$ in concentrated HCl. Magnesium was precipitated out as $Mg(OH)_2$ with an excess of 1N NaOH. The precipitate was dissolved with 1N H_2SO_4 and the Mg was made to a final concentration of 0.4 meq per ml.

To determine the exchangeable magnesium content (Mg_e) of the body, each animal was given 5 ml of this solution (a total of 2 meq of Mg and 10 microcuries of Mg^{28}) by intravenous injection. The urine was collected for 24 hours and pooled. Between the 24th and 28th hours, 2 spot specimens were collected by catheterization and their specific activities were determined. The Mg_e value was calculated using the following formula:

$$Mg_e = \frac{Mg_i^{28} - Mg_o^{28}}{Mg_u^{28}/Mg_u^{24}}$$

Mg_i^{28} = quantity of radiomagnesium administered (arbitrary units).

Mg_o^{28} = quantity of radiomagnesium excreted in the urine up to the time the spot specimens were obtained.

Mg_u^{28} = concentration of radiomagnesium in spot specimen.

Mg_u^{24} = concentration of stable magnesium in spot specimen.

Mg_u^{28}/Mg_u^{24} = specific activity of spot specimen.

Mg_e = quantity of exchangeable magnesium in milliequivalents.

Samples of plasma, urine, and tissues were assayed for gamma ray activity with a well-type scintillation counter.

Magnesium concentration in urine and serum(4) and in tissues(5) was determined by a modification of the molybdivanadate method for phosphate.

To determine the external balance of magnesium, body weight, total food consumption, and 24-hour urine volume were measured each morning before the rabbits were fed.

Experimental procedure. Baseline measurements of body weight, food intake, and urinary excretion were made daily during the first 7 days. Starting on the 7th day and continuing every day for 8 days, 0.1 mg of digitoxin per kg body weight was given intravenously to each of 8 animals. The remaining 4 rabbits were not given digitoxin, and served as controls. Two baseline Mg_e determinations were

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* This work was supported by Contract between U. S. Atomic Energy Commission and Univ. of Colorado.

Mg^{28} was supplied by Brookhaven National Laboratory, Upton, L. I., on allocation from U. S. Atomic Energy Commission.

made on the 1st and 7th days of the experiment; the test measurement was made on the 16th day. Twenty-eight hours after completion of the third (test) Mg_e, the animals were killed by air embolism, and 2 samples of each tissue were obtained for radioactivity assay and determination of the magnesium content.

The 4 animals not receiving digitoxin were killed in the same fashion 24 hours after administration of Mg^{28}, and tissues were obtained for assay of radioactivity and magnesium content.

Statistical method. In the analysis of the balance data, each animal served as its own control. After the differences between the mean of the baseline values and the mean of the values obtained during the experimental period had been calculated for each animal, mean difference for the group was determined. The significance of the mean difference was obtained by the use of the "t" test(6), a "P" of less than 0.01 being considered significant.

Mean relative activity and tissue magnesium content of each type of tissue were determined for both test and control groups, and the significance of the differences between group means was tested(6).

Results. While no significant changes were found in the external balance study, the exchangeable body content of magnesium increased significantly in the animals injected with digitoxin (Table I). No significant changes were noted in the relative radioactivity of the tissues studied (Table II), although in every instance, mean values were higher in the test group than in the control group. A significant reduction in magnesium content was noted in skin of animals given digitoxin (Table III).

Comment. The results suggest that digitalis might induce some subtle changes in the dynamics of magnesium metabolism which are not reflected in the external balance of this ion. The significant increase in Mg_e, associated with an increase (even though a small one) in the mean values for the relative radioactivity of all the tissues studied, suggests that digitalis produced a generalized increase in the tissue uptake of Mg^{28}. The significant decrease in magnesium content of skin is difficult to interpret in the light of our present knowledge of the pharmacology of digitoxin.

TABLE I. Effects of Digitoxin, Given Daily for 8 Days in Doses of 0.1 mg/kg, on External Magnesium Balance, Exchangeable Magnesium, and Serum Magnesium in 8 Normal Rabbits

	Mean Baseline Values†	After Injection of Digitoxin
Weight (kg)	1.635	1.754
Serum Mg (meq/l)	2.0	1.6
Exchangeable Magnesium (meq)	51.5	82.1*
" " (meq/kg)	31.1	46.8*
Mg Intake (meq/day)	12.4	15.0
Urine Mg Excretion (meq/day)	6.3	5.9
" Volume (ml/day)	141	135

† Mean value for 7 days.

* Statistically significant difference when compared with the mean baseline value.

TABLE II. Effects of Digitoxin on Relative Radioactivity of Tissues in 8 Rabbits

Tissue	Relative Radioactivity† Control Group‡	Test Group§
Muscle	2.06 ± .16‖	2.77 ± .35
Skin	3.28 ± .13	3.38 ± .24
Kidney	8.16 ± .38	9.33 ± .65
Heart	9.46 ± .55	10.01 ± .68
Liver	10.02 ± .62	12.74 ± 1.47
Appendix	12.26 ± .67	14.82 ± .64
Bone	33.73 ± 2.47	39.72 ± 3.62

† Relative radioactivity $= \frac{\text{cpm/gm tissue}}{\text{cpm/ml plasma}}$ 24-28 hr after intravenous injection of Mg^{28}.

‡ Mean values in 4 rabbits not given digitoxin.

§ Mean values in 8 rabbits given intravenous injection of digitoxin, 0.1 mg/kg, daily for 8 days.

‖ Mean ± standard error.

TABLE III. Effects of Digitoxin on Tissue Content of Magnesium in 8 Rabbits.

Tissue	Magnesium Content (meq/kg wet wt) Control Group†	Test Group‡
Muscle	21.5 ± .15§	19.8 ± .83
Skin	6.4 ± .27	5.0 ± .32*
Kidney	12.2 ± .66	11.9 ± .41
Heart	13.0 ± .40	13.7 ± .41
Liver	12.4 ± .50	14.9 ± .98
Lung	11.8 ± .76	14.5 ± 2.69

† Mean values in 4 rabbits not given digitoxin.

‡ Mean values in 8 rabbits given intravenous injection of digitoxin, 0.1 mg/kg, daily for 8 days.

§ Mean ± standard error.

* Statistically significant difference when compared with control group.

It has been previously suggested that digitalis might have pharmacologic effects similar to those of the adrenal cortical steroids. Cortisone and digitalis both tend to deplete the body store of potassium(3,7). In rabbits cortisone produces an increase in the Mg_e, accompanied by an accelerated uptake of Mg^{28} in muscle, appendix, and heart(8). Digitalis, in the present study, produced similar changes in magnesium metabolism. Since it is difficult to explain these findings solely on the basis of the cardiotonic effects of digitalis, the results of the present study furnish additional evidence that this drug also has an extracardiac action.

Summary. In a test group of 8 rabbits, administration of digitoxin by intravenous injections of 0.1 mg/kg, given daily for 8 days, produced no significant changes in the external balance of magnesium. On the day following the last injection the exchangeable body content of magnesium, measured with Mg^{28}, was significantly increased. The results suggest that digitoxin produces subtle changes in the dynamics of magnesium metabolism which are not reflected in the external balance data.

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Use of Radioiodinated Antipyrine to Measure Liver Function in the Rat.* (27035)

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Radioiodinated antipyrine (RIAP), when injected in the rat, releases significant quantities of its I^{131} tag within one hour. The I^{131} tag appears in the peripheral blood in an increasingly diffusible form. This phenomenon can be measured by the rate of dialysis of radioactivity from plasma through a cellophane membrane(1). Brodie and Axelrod(2) have shown that in man, 30% of a dose of antipyrine is hydroxylated by the liver at the 4′ position, immediately conjugated with glucuronic or sulfuric acid and excreted in the bile. It has been suggested that since the I^{131} tag of RIAP is located at the 4′ position, its separation from the antipyrine molecule was a function of the liver(1). In the experiments reported here, liver function was altered by 3 different methods in rats. RIAP was then administered to these and to control animals. A plasma sample was obtained after one hour, and the amount of radioactivity remaining in the sample after a one hour dialysis was determined. The results indicate that impairment of liver function in the rat greatly reduces the loss of I^{131} tag from RIAP.

Methods. Young adult Sprague-Dawley rats, weighing 150-200 g were used in all experiments, male rats in Exp. I and II, females in Exp. III. 20 μc of RIAP (Abbott) in 0.5 ml 0.9% saline was administered intravenously to each animal. In all animals, anesthesia was induced with 40 mg/Kg sodium pentobarbital, given intraperitoneally.

In Exp. I, male rats were divided into experimental and control groups and allowed food and water *ad libitum* until anesthetized. The experimental animals were then eviscerated after ligating and severing the celiac axis and portal vein. The liver was left *in situ*, after

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the technique described by Russel(3). RIAP was then injected into the inferior vena cava and the incision closed by wound clips. After one hour, blood samples were obtained by aortic catheterization. The blood was centrifuged and 0.5 ml plasma samples placed into counting tubes. One ml of plasma was then placed in a cellophane bag (80 μ thick, pore size 30 Å) suspended in 20 ml of distilled water and the dialysis allowed to proceed for one hour(1). At the end of this time, 0.5 ml samples were taken from inside the dialysis bag. All samples were then counted in a well-type scintillation counter and the percent of radioactivity remaining inside the dialysis bag calculated.

In Exp. II, male rats were separated into experimental and control groups and fasted for 12 hours with free access to water. The experimental animals were then given an intraperitoneal injection of 0.3 ml/100 g of a 1:10 mixture of carbon tetrachloride USP and mineral oil, according to the method of Koch-Weser, Farber and Popper(4). Twenty-four hours after this injection, the animals were anesthetized, laparotomies performed and RIAP injected. One hour later blood samples were obtained and treated as in Exp. I.

In Exp. III, female rats were divided into 3 groups and fasted for 12 hours. The rats comprising Group 1 were then given an intraperitoneal injection of 75 mg/100 g of an aqueous solution of dl-ethionine in 3 divided doses(4). Animals Group 2 were given carbon tetrachloride as described above. The animals in Group 3 served as controls. After 48 hours, the animals were anesthetized and treated as described in the preceding experiments.

Results. The data from the 3 experiments are given in Table I and shown graphically in Fig. 1. After dialysis, the percent of radioactivity remaining in plasma samples from control rats in Exp. I and II, with unimpaired liver function, remained within a narrow range (5.6-10.6%). In Exp III, after 60 hours of fasting, a higher percentage of radioactivity remained (P<.01, Fig. 1). All methods utilized to impair liver function resulted in a significantly higher percentage of radioactivity remaining in the plasma of experimental animals after dialysis. The least loss of I^{131} from RIAP was seen in those animals with ligation of the celiac axis and portal vein. Acute poisoning with carbon tetrachloride or with dl-ethionine resulted in percentages lower than the experimental group of Exp. I, but significantly higher than in control groups. Significantly higher percentages were observed 24 hours after carbon tetrachloride poisoning than after 48 hours.

TABLE I. Results of dialysis of radioactivity from rat plasma after injection of radioiodinated antipyrine.

Exp.	No. Rats	% Radioactivity remaining in plasma after 1 hr dialysis ± S.D. (range)
I A Evisceration	10	26.5 ± 5.8 (17.7 – 32.9)
I B Control	10	7.8 ± .5 (5.0 – 10.6)
II A CCl_4 24 hr	6	18.5 ± 1.7 (16.5 – 21.3)
II B Control	6	8.2 ± 1.0 (6.8 – 9.2)
III A Ethionine	7	16.5 ± 3.9 (9.8 – 21.8)
III B CCl_4 48 hr	6	14.3 ± 2.6 (11.1 – 18.6)
III C Control	8	9.1 ± 1.5 (7.5 – 12.4)

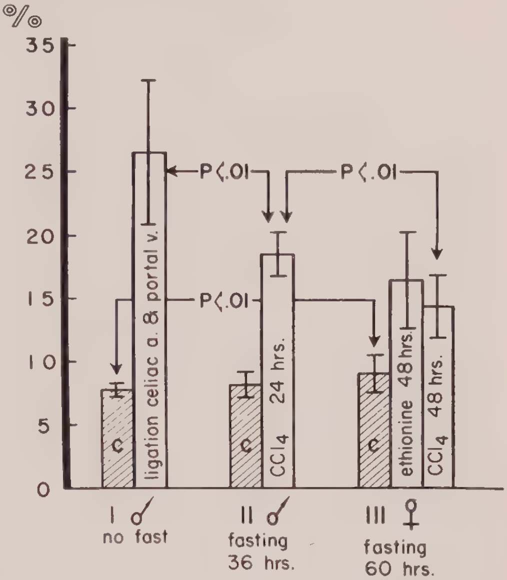

FIG. 1. Chart showing percent radioactivity remaining inside the cellophane membrane after 1 hr. dialysis of plasma from experimental and control (c) rats. Vertical bars represent S.D.

Discussion. This study shows that impairment of liver function results in a decrease in diffusibility of radioactivity through cellophane in the plasma of the rat following injection of RIAP. This decrease can be detected by a relatively simple procedure since it is correlated with the percent radioactivity remaining in a plasma sample after dialysis.

The comparatively narrow ranges of values observed in the control animals of Exp. I and II indicated that the amount of freely diffusible radioactivity appearing in the plasma of rats one hour after administration of RIAP is relatively constant. This range was taken to indicate normal liver function. The less diffusible form of radioactivity observed in the control animals of Exp. III differed significantly from this range, indicating impairment of liver function after a 60 hour fast. It has been shown by Addis *et al.*(5) that the fasting rat loses 20% of its liver protein after 48 hours, and 40% after 168 hours. Miller(6) has demonstrated a loss of liver catalase, alkaline phosphatase, xanthine dehydrogenase and cathepsin activity in the fasting rat, which parallels or exceeds the loss of liver protein. A decrease in deaminating, transaminating and glycogen forming functions in protein-depleted rats has been described by Kaplansky et al(7). The present study indicates that along with the above impairment of enzymatic activity, there is an impairment of ability of the fasting rat liver to release I^{131} tag from the RIAP molecule.

It was noted that more severe forms of liver damage, such as that produced by ligation of its blood supply, produced the greatest decrease in cellophane diffusibility of the I^{131} tag, although the lesser degree of liver damage produced by carbon tetrachloride and dl-ethionine poisoning also resulted in a marked decrease in diffusibility.

Russel has reported that ligation of the celiac axis and portal vein results in total impairment of liver function in the rat(3). The results obtained in these eviscerated rats differed significantly from those rats poisoned with carbon tetrachloride or dl-ethionine. A statistically significant difference was also noted between the results in those animals studied 24 hours after carbon tetrachloride poisoning and those studied at 48 hours, reflecting the greater degree of liver function that has been reported to take place at this time(4). These data demonstrate that varying degrees of liver damage can be quantitated by this method.

Koch-Weser, Farber and Popper(4) measured liver function at several intervals following carbon tetrachloride and dl-ethionine poisoning, using the doses described above. At 48 hours they reported the following: control rats; BSP retention in mg % of serum, 0.12 ± 0.130; bilirubin 2.10 ± 0.64 mg %. Two days after ethionine poisoning; BSP 4.05 ± 0.980 mg % (80% retention).

It is concluded that impairment of liver function can be detected and quantitated by measuring the cellophane diffusibility of the I^{131} tag one hour after intravenous injection of RIAP in the rat. It is possible that this procedure can be adapted as an accurate measure of liver function in human subjects.

Summary. To determine whether impairment of liver function interferes with release of I^{131} tag from radioiodinated antipyrine, groups of rats were functionally hepatectomized, poisoned with carbon tetrachloride or poisoned with dl-ethionine. RIAP was then injected intravenously and the diffusibility of the I^{131} tag through a cellophane membrane measured after one hour. Functional hepatectomy resulted in a marked decrease in diffusibility, while carbon tetrachloride and dl-ethionine poisoning produced a significant decrease in diffusibility. Return of liver function after carbon tetrachloride poisoning resulted in an increase of diffusibility. The data support the conclusion that the release of I^{131} tag from RIAP is a function of the liver and can be used to measure liver function.

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Influence of Sex on Bone Marrow Transplantation in Mice With Induced Tolerance. (27036)

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Eichwald and Silmser(1) and Hauschka(2) reported that female mice frequently reject male skin grafts, while males usually accept female grafts. This phenomenon which was thought to be related to the sex chromosomes has been shown to play a significant role in every strain of mice tested(3,4,5,6,7,8,). Furthermore, using tolerant rat mice combinations, Billingham and Silvers demonstrated that this sex factor is also present in heterologous combinations, and added evidence to relate it to the Y chromosome(9). Rejection or decreased receptivity by female mice of male material was later reported in iso and homografts of transplantable tumors(10) and also in isologous normal thymus transplants(11).

Lengerova and Chutna(12) reported the appearance of secondary disease in C_{57}Bl males protected against lethal irradiation with isologous female bone marrow. Similar results were obtained by us in C_3H mice(13). They interpreted this secondary disease as being caused by the reaction of the female graft against the male host in a way equivalent to the "Eichwald and Silmser phenomenon," lending support to the graft-anti-host theory(14). During studies in our laboratory of the effect of induced "tolerance" on homologous bone marrow transplantation(15), it was observed that "tolerant" males behaved differently from "tolerant" females, when used as donors, or hosts, in homologous bone marrow transplantation experiments. This different behavior was thought to be related to the "Eichwald-Silmser phenomenon."

Material and methods. Newborn animals of A/Jax and C_3H strains† were injected, within 24 hours after birth with 2 to 5 × 10^6 bone marrow cells from C_3H and A/Jax adult females respectively, via the anterior fascial vein. The cells were obtained from marrow of a resected femur and suspended in 0.05 ml of 0.1% solution of Sequestrene in phosphate buffered saline at pH 6.5-7.2. The newborn were allowed to nurse under usual animal farm conditions and survivors were weaned at 30-40 days. At 6 weeks of age, a number of the injected C_3H animals were grafted with A/Jax skin (males with male skin and females with female skin). The animals which were used later for bone marrow transplantation experiments were irradiated and protected with marrow injections. The irradiation was carried out at 14-16 weeks of age by giving 800r in a rotatory lucite cage to groups of 10 animals, at 250kv, 15A, 4.0 mm. Al. filter, target distance 60 cm, rate 114-118r per minute. The adult animals which received marrow were given 10×10^6 cells in 0.5 ml of suspension, via tail vein, within 4 hours after irradiation. The animals were observed and weighed daily.

Results and discussion. Experiment 1. C_3H mice of both sexes, injected at birth with female A/Jax bone marrow, when grafted with A/Jax skin (males with male skin and females with female skin) uniformly rejected these skin-grafts; however, date of rejection was delayed as is shown in Graph 1. Examination of this data by Analysis of Variance (Appendix A) demonstrates that the injected animals rejected their grafts 3.7 days later than the non-injected controls ($P<0.001$) and there is no significant difference between sexes. These results are markedly different from those reported by Billingham and Brent(16,17) for this same strain combination. They have reported that 70% of their animals became at least partially tolerant and 43% of them were highly tolerant, while our animals as a group showed a slight degree of tolerance and none of them appeared to be highly tolerant. However, in their experiments, donors of the bone

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APPENDIX A. Analysis of Variance.

Source of Variation	Source of Squares	Degrees of Freedom	Mean Square	F	P (F)
Sex	2.74	1	2.74	.75	NS
Injection	179.43	1	179.43	48.89	.001
Sex-Injection Interaction	1.30	1	1.30	.35	NS
Individual Mice	213.04	58	3.67	—	—
Correction for Disproportion	34.26	—	—	—	—
Total	430.77	61	—	—	—

1. Effect or lack of effect of injection seems to be independent of sex of animals (no significant interaction between sex and injection).

2. No significant difference between sexes (in either non-injected or injected groups).

3. Highly significant effect of injection (regardless of sex). Injection associated with an average increase of 3.7 days in survival time of grafts.

marrow used to inject newborns were mostly males(17) whereas we have used females throughout.

Experiment 2. In a group of experiments to be reported elsewhere(15) we have observed a delay in the occurrence of secondary disease when C_3H females injected at birth with female A/Jax bone marrow cells were used as donors, or hosts, in homologous transplantation experiments. This was interpreted to mean that a slight tolerance (demonstrated in littermates by skin grafts), delays the immunological phenomenon which results in secondary disease.

In Graph 2 are plotted average body weight and survival curves of the following groups:

A) Eleven normal C_3H *males* lethally irradiated at 14-16 weeks of age and then protected with bone marrow from A/Jax *males* which at birth had received bone marrow cells from C_3H *females*. (tolerant donor, males).

B) Eleven normal C_3H *females* were lethally irradiated and then protected with bone marrow from A/Jax *females* which at birth had received C_3H *female* bone marrow(15). (tolerant donor, females).

C) Thirty male and female C_3H mice lethally irradiated and protected with male and female bone marrow respectively, from *normal non injected* A/Jax mice (males and females in this combination behaved identically, and are plotted together).

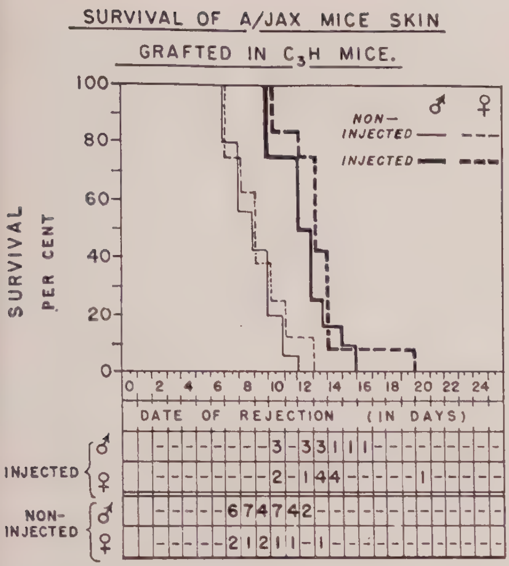
Paradoxically, the use of supposedly "tolerant" male donors, instead of increasing survival time of the male recipients, had the opposite effect: mean survival time of the Group A), 10.55 ± 6.4 days, is significantly shorter than the homologous bone marrow control C) (24.77 ± 12.3 days) ($P<0.01$), and much shorter than mean survival time of the females B) which received "tolerant" bone marrow (35.63 ± 13.9) ($P<0.001$). The survival curve of these males follows closely those reported in homologous experiments in which hypersensitized animals have been used(18,19,20).

The difference between Groups A) and B) is sex, and the paradoxical effect in A) is obtained by male donors which received female material at birth. It can be speculated that injection of animals at birth produced a "chimera"; if so, the A/Jax male donors of group A) contain C_3H *female* cells which have been sensitized against their *male* host, and A/Jax male cells which have become tolerant to female C_3H cells. When the bone marrow from these animals is used to protect normal C_3H *males*, their A/Jax cells will not react immediately against C_3H (the reaction will rather be delayed since the animals are partially tolerant), but the already hypersensitized C_3H female cells will react against "male."

Experiment 3. In Graph 3 are plotted the average body weight and survival curves of the following groups:

D) Eight C_3H *males* which at birth had received bone marrow from A/Jax *female* donors, were lethally irradiated (at 14-16 weeks of age) and protected with A/Jax male bone marrow. ("tolerant hosts" males).

E) Thirteen C_3H *females* which at birth had received female A/Jax bone marrow (littermates of Group D), were irradiated and protected with normal A/Jax female bone marrow. ("tolerant hosts" female).



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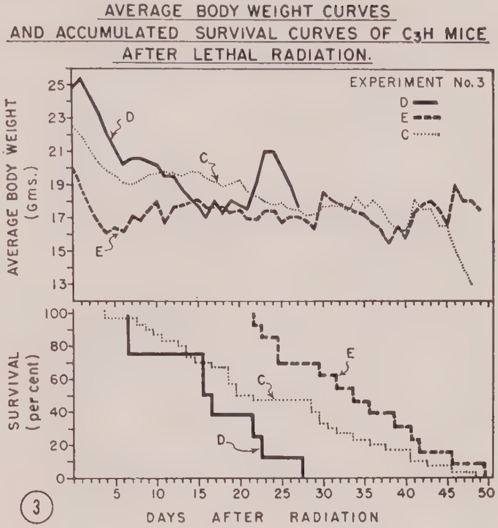

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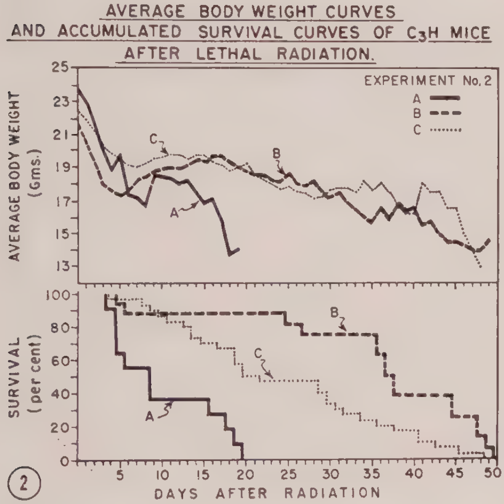

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FIG. 1. Survival and date of rejection of grafts of A/Jax skin in C3H mice — injected at birth with A/Jax bone marrow—as compared with similar grafts in non-injected C3H mice. Data for both sexes are plotted separately.

FIG. 2. Average body weight curves and accumulated survival curves of C3H mice after lethal irradiation and protection. A=C3H males protected with bone marrow from A/Jax male mice—injected at birth with C3H female bone marrow—("tolerant" donor, males). B=C3H females protected with bone marrow from A/Jax female mice—injected at birth with C3H female bone marrow—("tolerant" donor, females). C=C3H mice (males and females) protected with bone marrow from normal non-injected A/Jax mice (males and females respectively).

FIG. 3. Average body weight curves and accumulated survival curves of C3H mice after lethal irradiation and protection. D=C3H males—injected at birth with A/Jax female bone marrow—protected with bone marrow from normal non-injected A/Jax males ("tolerant" hosts, males). E=C3H females—injected at birth with A/Jax female bone marrow—protected with bone marrow from normal non-injected A/Jax females ("tolerant" hosts, females). C as in Fig. 2, C.

C) The same homologous bone marrow control as in Exp. 2. Mean survival time for the males D) (17.00 ± 7.4 days) is shorter than that of the homologous bone marrow controls, (24.77 ± 12.3 days) but this difference does not reach statistical significance. However, when this mean survival time is compared with that of the "tolerant" female Group E), (34.23 ± 9.1 days), the survival of the males is significantly shorter ($P < 0.001$).

As in the previous experiment, it may be postulated that the injected animals are partial chimeras and that the female cells they carry are already hypersensitized against males. If this assumption is correct, this hypersensitization is responsible for the shortening of survival in the males.

Summary. Mice of A/Jax and C_3H strains were injected at birth with bone marrow cells from females of the opposite strain in an at-

tempt to induce "tolerance." Skin grafting experiments demonstrated that both males and females so treated did acquire a slight degree of tolerance. When the partially tolerant female mice were used as donors or hosts in homologous bone marrow transplantation experiments (with the opposite strain), the date of appearance of the secondary disease was delayed, an effect attributable to acquired tolerance. However, when partially tolerant males were used, survival did not increase, but actually decreased. The animals died in a very short time, as if hypersensitized. It has been proposed that the "tolerant" animals are chimeras; if so, the male animals may contain female cells already hypersensitized against "male," and these "hypersensitized" cells may be responsible for the short survival.

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A Goitrogenic Rat Diet Producing Normal Body Weight Gain.* (27037)

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The first demonstration of low iodine goiter in the rat was reported by Levine, Remington and Von Kolnitz(1), and the diet they designed is, with minor modifications, still in general use today and has come to be known as the Remington diet. Attempts to improve the slow rate of body weight gain produced by this diet by adding animal proteins(2) have shown that such additions reduced the goitrogenic effect of the diet, making it less suitable for use in experimental goiter studies. Thus Parker *et al.*(3) observed good growth in rats fed a diet similar to the Remington diet with addition of DL-methionine, dried beef and "animal protein factor," but only the second generation of animals showed substantial thyroid enlargement, whereas growing rats consuming the standard Remington diet consistently develop goiter in 5 weeks.

The present paper describes a new low iodine goitrogenic rat diet, formulated without animal proteins, that supports a normal rate of body weight gain.

Materials and Methods. Brewers type dry yeast (iodine 0.06 μg/g) at 40% by weight of the diet, supplemented with DL-methionine at 0.5% by weight of the diet, was employed as the principal protein source in the diet coded YST in Table 1. To provide comparisons with a low iodine semipurified diet containing an animal protein, the diet coded CAS in Table 1 was designed to contain casein at a level of 20% by weight. The remaining ingredients of these diets are listed in the table footnotes.

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The Remington diet (code REM) was also included in the experiment. The formula employed was the standard one(2), modified only to include a complete vitamin mixture, in order to match diets YST and CAS in this regard. Finally, a commercial rat chow (coded PUR) was fed to provide data from "normal" animals. Thus 3 low iodine diets of known composition, one containing an animal protein, were fed in parallel with a widely used rat chow whose quantitative composition is not revealed but which contains a variety of animal proteins and an ample amount of iodine.

Male albino rats, descendants of the Sprague-Dawley strain, were weaned at 3 weeks of age, when their body weights ranged from 45 to 55 g, and averaged close to 50 g. The rats were held in separate cages with wire mesh bottoms, were provided *ad libitum* with demineralized water from an ion exchange column, and were fed one of the 4 experimental diets *ad libitum* for 7 weeks, while records of individual food intake and body weights were kept. Then the animals were killed, and the fresh weights of their thyroid glands determined using an analytical balance. Iodine in diets and in thyroid glands was determined by the method of Chaney(4).

Results. In Table 1, the weight gained by the rats fed the commercial chow may be taken as "normal." The Remington diet supported, as expected, about half the normal rate of body weight gain. The 2 semipurified diets YST and CAS, to the contrary, produced approximately normal rates of gain.

Goitrogenic effects may be judged by comparing thyroid gland/body weights, and also by comparing iodine contents of the pooled thyroid glands. The data shown for animals fed the Remington diet are concordant with prior publications(5), and may be taken as representative values for simple low iodine goiter in this species. The thyroid gland data from rats fed the commercial chow may be taken as "normal," since they agree with prior values for this sex and strain of rat fed diets adequate in all nutrients including iodine(6).

Thus all 3 low iodine diets produced enlarged thyroid glands with lowered iodine contents. Within the limitations of the iodine assay methods at this low level(7), diet CAS had about the same low iodine content as diet REM; but the animals fed diet CAS nevertheless had significantly smaller thyroids (P = .025), containing 3 times as much iodine, as rats fed diet REM. That this effect was due to the casein content of the diet CAS is suggested by numerous uncontroverted reports that ca-

TABLE I. Effects on Thyroid Parameters of Various Diets Fed Ad Libitum to Male Sprague-Dawley Strain Rats* for 7 Weeks Starting at 3 weeks of age. Average Values.

	DIET CODES†			
	YST	*REM*	*CAS*	*PUR*‡
No. of Rats	6	10	10	9
Iodine in Diet, μg/g	.03	.001	.009	2
Food Consumed, g	672	636	868	§
Wt Gained, g	213	117	245	227
Thyroid Wt mg/100 g :Avg	25.2	19.3	15.3	7.6
: St. Dev.	5.6	4.5	2.5	.8
Iodine in Pooled Thyroids, % of Dry wt	.0043	.0097	.0300	.2000

* Purchased from S & K Animal Co., Jamesburg, N. J.

† Ingredients of diets in % by weight: Diet *YST;* ground whole yellow corn 45.3, dry brewers type (Fleischmann's 50B) yeast 40, corn oil 8, salt mixture (USP XIII No. 2) 4, complete vitamin mixture triturated in dextrose (Nutritional Biochemicals Corp.) 2.2, DL-methionine 0.5. Diet *REM;* corn 75.8, wheat gluten 18, dry yeast 2, calcium carbonate 1, sodium chloride 1, vitamin mixture 2.2. Diet *CAS;* purified casein 20, corn starch 47.8, cellulose 14, corn oil 12, salt mixture 4, vitamin mixture 2.2.

‡ Purina Laboratory Chow, Ralston Purina Co., St. Louis Mo.

§ Not recorded.

sein has antigoitrogenic properties(8). Precisely the reverse may be seen for the data from rats fed diet YST. Their mean thyroid weight was significantly greater than those of rats fed diet REM (P=.05). Furthermore, their thyroid iodine content was only half as great as those of animals fed diet REM. Thus the low iodine 40% yeast diet, which had supported normal growth, did not exert an antigoitrogenic effect as do low iodine casein diets such as diet CAS. Indeed in this experiment diet YST had a significantly positive goitrogenic effect, over and above that to be expected from its low iodine content.

Discussion. Dry yeast has been used frequently in experimental animal diets, but rarely if ever at levels as high as the 40% employed in this study. This high level of yeast, together with supplementation with methionine, enabled diet YST in the present study to support a rate of gain in body weight similar to that supported by a standard laboratory rat chow or a semipurified diet based on casein. As the dry yeast had a low iodine content (0.06 μg/g), it was possible to use it in a low iodine diet. Furthermore, as seen in this study, the yeast did not have an "antigoitrogenic" effect aside from its iodine content, such as has been attributed to animal proteins like casein.

Hence diet YST, or similar ones, may prove useful in experimental goiter studies with the rat. The literature on dietary goitrogenesis does not clarify the apparent positive goitrogenic effect, over and above that attributable to low iodine content, seen with diet YST in this study.

Summary. By using 40% of brewers type dry yeast as the principal protein source, a low iodine diet has been designed that supports a good rate of body weight gain and also produces large goiters in rats. This diet appears to have a positive goitrogenic effect, over and above that attributable to its low iodine content.

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Effect of Dilantin Sodium on Various Cell Lines in Tissue Culture.* (27038)

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Previous studies from this laboratory(1,2) have clearly shown the dramatic stimulatory effects of sodium 5,5 diphenylhydantoin (Dilantin Sodium) and certain related analogues on the growth of a human gingival fibroblast-like cell in tissue culture. This property is consistent with the Dilantin-induced stimulation of wound healing in rats as judged by increased tensile strength also reported by this laboratory(3) and with well-known gingival hyperplasia occurring in epileptic patients under treatment with this drug. During these studies, it was found that the HeLa cell in tissue culture responded to Dilantin by a very limited stimulation of proliferation but there was no significant stimulation of cells isolated from rat fibrosarcoma. This variability of cell response suggested that this stimulatory effect of Dilantin might be quite cell-specific and, for this reason, a series of different cell lines carried in this laboratory have been tested.

Materials and Methods. Two general types of cell lines have been tested: those isolated from 1) normal tissue and 2) tumor tissue (Table

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* Supported by research grant from Nat. Inst. of Dental Research, U.S.P.H.S.

I†). In these studies, actively growing serial transfer cultures of cells were trypsinized, washed, suspended in their appropriate growth media and dispensed into culture tubes. The growth medium for each cell line was not identical but usually differed only in type and concentration of blood serum (calf or horse) and the variable presence or absence of such constituents as lactalbumin hydrolysate and egg ultrafiltrate. The medium in the experimental tubes was identical with that in control tubes for each cell line in all cases except that, in addition, the experimental medium contained 200 μg/ml of Dilantin.**

After incubation at 37°C in a stationary position, either 2 or 3 tubes were picked at random from each group at the varying time intervals shown and cell population determined by counting viable resuspended cells, stained with trypan blue, in a hemacytometer. The complete technique for these various procedures has been reported(1,2).

Results and Discussion. The results of these studies are shown in Table I. A single test series on the human gingival fibroblast-like cell and on the HeLa cell, both previously reported (1), are shown for comparative purposes. Only data for a single test series are shown for each cell line. However, in every case the findings in each series were consistent with respect to the magnitude of cell response to the drug. The individual data shown were carefully selected to be respresentative of the various series of studies carried out on each cell line.

The data indicate that a distinctive selectivity exists in the stimulatory effect of Dilantin on different cells in tissue culture. Of these cells tested derived from normal tissues, only fibroblasts (the human gingival fibroblast isolated in this laboratory and Earle's L929 mouse fibroblast) responded to the drug by a dramatic growth stimulation. Epithelial cells or cells derived from ectodermal organs demonstrated no remarkable response to Dilantin. Cells derived from adult mouse heart demonstrated a moderate stimulation in response to Dilantin at a 7 day period. However, this culture, even after 125 transfers appears to consist of 2 types of cells, and the stimulated cell bears a great resemblance to a fibroblast.

Of cell lines derived from tumor tissues tested, only the BW1081 mouse salivary gland tumor showed a similar dramatic stimulatory response. This is somewhat surprising, as this tumor was originally presumed to be of epithelial origin. However, evidence reported by Beck suggests that this tumor may be related to the polyoma virus tumor(4). Furthermore, reintroduction of these tumor cells grown in tissue culture for over 155 serial transfers into the BALB/c mouse in this laboratory has resulted in tumor formation but, interestingly, of a somewhat different microscopic pattern, more suggestive of a fibrosarcoma than the original epithelial neoplasm. The significance of this finding is not apparent although sarcomatous transformation of epithelial cells is rather common in tissue culture and has been discussed by Sandford et al(5).

The HeLa cell, as previously reported, continued to show a moderate stimulation of cell proliferation but of far less magnitude than either normal fibroblasts or the BW1081 tumor. Upon further testing the rat fibrosarcoma also showed a stimulation of the same approximate magnitude as the HeLa cell. Furthermore, the H-P melanoma line showed a response quite comparable to the normal adult mouse heart, the rat fibrosarcoma and HeLa cell. Despite the fact that these latter cell lines showed a statistically significant ($p = < 0.01$) stimulation of prolifieration in response to Dilantin, this cannot be considered of biologic significance equal to the dramatic response shown by the 2 lines of normal fibroblasts and the BW1081 tumor.

The data definitely suggest that the stimulatory effects of Dilantin are specific for fibroblasts. These findings are consistent with the report of Houck *et al*(6) that administration of Dilantin to rats resulted in changes in skin chemistry consisting of a marked increase in insoluble collegen and insoluble hexosamine, and the appearance of a unique insoluble noncollagenous protein, all implying a marked al-

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† The human squamous epithelium utilized in these studies was courteously supplied by Dr. Irving S. Johnson, Eli Lilly Co., Indianapolis, Ind. The Earle's L929 fibroblast was purchased from Microbiological Associates, Bethesda, Md. The Henle's epithelial cell was courteously supplied by Dr. J. Donald Hubbard, Indiana Univ. School of Med., Dept. of Pathology.

** Generously supplied by Parke, Davis & Co., Detroit, Mich.

TABLE I. Effect of Dilantin Sodium on Growth of Various Cell Lines.

Cell Line	Mean Cell Count (per ml of medium) x 10^5 Interval (days) 0	1	4 or 5	7 or 8	% stimulation of cell proliferation (4 or 5 days)
Normal tissues					
1) Adult mouse heart(3)*					
Control	.78	.83	2.00	3.83	18
Dilantin	.81	.83	2.36	4.66	—
2) Adult mouse kidney(3)					
Control	1.01	1.16	3.85	5.19	0
Dilantin	1.01	1.15	3.67	5.08	—
3) Adult mouse liver(2)					
Control	.97	1.04	2.68	4.04	1
Dilantin	.97	1.05	2.71	4.03	—
4) Newborn mouse lung(2)					
Control	.98	1.03	2.85	4.06	—
Dilantin	.97	1.04	3.03	4.08	7
5) Henle's intestinal epithelium(2)					
Control	.70	.71	2.11	3.49	—
Dilantin	.71	.70	2.15	3.49	2
6) Human squamous epithelium(2)					
Control	.74	.78	1.99	3.40	—
Dilantin	.78	.81	2.00	3.48	0
7) Earle's L929 mouse fibroblast(2)					
Control	.74	.79	1.99	3.39	—
Dilantin	.74	.81	2.99	4.28	50
8) Human gingival fibroblast-like cell(18)†					
Control	1.01	1.18	2.23	4.00	—
Dilantin	1.01	1.20	4.26	6.04	91
Tumor Tissues					
1) Rat fibrosarcoma(3)					
Control	.94	1.13	4.02	5.36	—
Dilantin	.94	1.13	4.69	5.99	17
2) Rat rhabdomyosarcoma(2)					
Control	1.01	1.14	4.09	—	—
Dilantin	1.01	1.14	4.04	—	0
3) H-P melanoma(2)					
Control	.68	.88	2.13	2.71	—
Dilantin	.70	.86	2.46	3.08	16
4) HeLa Cell(3)†					
Control	1.02	1.16	1.78	6.05	—
Dilantin	1.00	1.26	1.99	6.86	12
5) BW1081 mouse salivary gland tumor(5)					
Control	.92	1.05	3.36	5.80	—
Dilantin	.90	1.05	6.86	7.35	104

* Figures in parentheses indicate minimum number of times cell line was tested.
† From previously reported studies(1,2), for comparative purposes.

teration in fibroblast metabolism.

The significance of these findings is manifold. They suggest not only a technical method for separating fibroblasts from other contaminating cells in tissue culture, but also a possible means for better understanding of the metabolism of the fibroblast.

Summary. A variety of cell lines, derived from both normal and tumor tissues, has been tested for possible stimulatory effects induced by sodium 5,5 diphenylhydantoin (Dilantin Sodium). Of these cells utilized, only fibroblasts responded by a stimulation of proliferation with the exception of the BW1081 mouse salivary gland tumor. The significance of these findings may be of considerable importance in our ultimate understanding of fibroblast function.

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Response of Bone Sodium to Acute Changes in Serum Sodium: Effect of Age.† (27039)

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Previous work in our laboratory has failed to show more than a modest decrease in bone sodium content in response to acutely induced changes in extracellular fluid composition(1, 2,3). Most of the experiments reported in the literature have involved a combination of acidosis and hyponatremia, including those of Bergstrom, who was the first to suggest that sodium could be readily mobilized from bone (4,5). Only a few attempts have been made to study the effects of alteration of serum sodium concentration alone. Acute hyponatremia is reported to result in a drop of 0-7% in bone sodium(1,3,6,7,8,9), though Woodbury(10) reports a drop of 19%, while acute hypernatremia fails to cause a rise. Most of these studies have been done on adult animals, and in no instance was the induced change in serum sodium a large one.

Two considerations suggested the possibility that changes might be produced more readily in young animals. Bergstrom found that a combination of acidosis and hyponatremia produced a larger decrease in bone sodium in younger animals. Forbes *et al*(11) and Munro *et al*(12) reported that radiosodium exchange occurred more rapidly in young bone. We therefore undertook a study of rats of different ages with respect to the effects of acute hypo- and hypernatremia.

Methods. Albino rats aged 20, 40 and 90 days were used. The 40 and 90 day old animals (males) were fasted overnight but allowed water. The 20 day old animals (mixed sexes) were removed from their mothers one day prior to experiment and fed Purina fox chow. They were fasted only during the experiment. An attempt was made to select animals of comparable weight in each group.

Approximately one-third of the animals in each group served as *controls*. One-third were rendered *hypernatremic* by repeated intraperitoneal injections of 2% NaCl (360 mM/l.). The injections were given hourly for 3-4 hours whereupon the animals were killed by decapitation. Two of the 40 day old animals became moribund within 2 hours, and were killed at that time. The volume given at each injection was 10% of body weight, so that the total sodium load was 110-140 meq/Kg, or substantially more than the total sodium content of the animal. The remaining animals were rendered *hyponatremic* by a similar treatment with 5% dextrose solution given intraperitoneally. Some leakage of peritoneal fluid occurred in these animals. Most of the animals appeared moribund at the end of the experiment. Blood for sodium analysis was obtained by decapitation.

Analysis of Bone. The long bones were used. Prior to dissection the lower extremities were dipped in distilled water to remove contamination with peritoneal fluid. The long bones were then removed, cleaned of periosteum and marrow, and the epiphyses clipped off. The resulting sample of marrow-free cortical bone was weighed as soon as possible. Analysis for water, ash, and sodium were made according to previously described methods(3).

Results are set forth in Tables I and II and Fig. I. The magnitude of the induced changes in serum Na (extreme range 66-246 meq/l.) is evident from the figure. In the control animals, bone Na and bone ash content progressively rose with age, while bone H_2O content fell, in accordance with previous work(3,11). Neither bone H_2O nor bone ash content changed significantly in the experimental animals, despite the large loads of water and salt administered.

No attempt was made to calculate average content of total bone sodium for the experimental groups in view of the variations in serum Na concentration which occurred. Rather, a plot of total bone Na against serum Na for each animal is included in Fig. I, to-

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TABLE I. Analytical Data for Bone Composition.

Group	No. Animals	Bone H_2O, %	Bone Na, meq/Kg wet wt Total	Crystal Phase	Bone Ash %	Serum Na meq/l
			20 days, 22-36 g			
Water Load	7	47		46	29.9	
		(44-49)*		±2†	±.8†	
Control	8	48	100	36	28.4	134
		(43-51)	±3†	±4	±.7	(131-138)*
Salt Load	6	46		40	29.5	
		(44-48)		±3	±.6	
			40 days, 80-110 g			
Water Load	5	27		126	48.4	
		(26-28)		±3	±.5	
Control	4	31	168	125	47.9	141
		(27-36)	±4	±3	±1.2	(137-154)
Salt Load	8	28		126	47.8	
		(24-32)		±4	±.8	
			90 days, 210-260 g			
Water Load	6	18		180	58.8	
		(15-21)		±8	±.8	
Control	6	19	205	177	57.7	144
		(14-22)	±4	±8	±.8	(138-148)
Salt Load	6	18		179	59.4	
		(16-20)		±6	±.6	

* range † Standard error

TABLE II. Calculated Data for Bone Composition.

Age Group days	Average Bone H_2O, %	Regression slope	y-axis intercept, meq/Kg	"Available" fraction	"Exchangeable" fraction†
20	46.9	.403	50	.52	.80
		±.031*			
40	28.2	.287	125	.25	.64
		±.034*			
90	18.5	.156	183	.11	.44
		±.045*			

* standard error; $p < .01$. † from previous data(3).

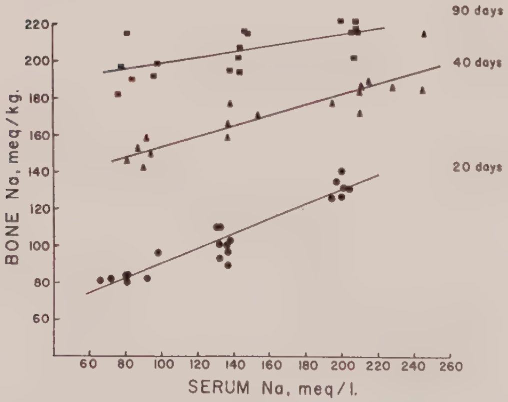

FIG. 1. Plot of bone sodium content against serum sodium for rats of varying age. Calculated regression lines are included.

gether with the calculated regression lines for each group. The bones of the hypernatremic animals contain more Na, and those of the hyponatremic animals less Na than those of the controls in each of the 3 age groups. Moreover, the regression slope becomes progressively less steep with advancing age. Thus the observed change in total bone Na resulting from a given increment or decrement in serum Na is a function of age.

The parameters of the 3 regression equations are listed in Table II. Each of the slopes is significantly different from zero.

The regression slope $\frac{(\triangle \text{ bone Na})}{(\triangle \text{ serum Na})}$ can be taken to represent the sodium "space" of bone; multiplication by 100 yields a value for percentage change in bone Na for each 100% change in serum Na. It is of interest to compare these values with those for bone water content. For the 20, 40 and 90 day old animals, respectively, Na "space" is 85.9%, 101.8%, and 84.3% (avg. 90.7%) of bone water. The standard error of these values (5.6%) is such as to permit the conclusion that calculated Na "space" does not differ significantly from measured bone water content.

Extrapolation of the regression line to zero serum Na (the y-axis intercept) yields values which agree fairly well with calculated "crystal" phase Na (Table I). "Crystal" phase Na was calculated by subtracting "fluid" Na (serum Na x bone H_2O) from total bone Na. Corrections for serum H_2O content and the Donnan factor were omitted since the 2 nearly cancel out. The chloride "space" of bone was not used since it has been shown that this often exceeds total bone water(3). Dosekun reports that inulin space is 57% of bone H_2O(9). No change occurred in "crystal" phase Na as a result of the experimental procedure; thus, the observed changes in total Na content must be ascribed to those taking place in the "fluid" phase.

A further calculation can be made; namely, the fraction of the total Na of bone which is subject to the effects of alterations in serum Na concentration. On the assumption that the relationship between total bone Na and serum Na is linear, the ratio of calculated sodium "space" Na to total bone Na at normal serum Na concentration is derived from the regression equation for each age group. This ratio is termed the "available fraction" in Table II. The magnitude of this fraction is clearly a function of the age of the animal.

Discussion. We have shown previously that radiosodium exchange is 2-3 times greater in weanling rat bone than in the adult. The present work shows an age effect of similar magnitude for that fraction of bone Na which responds to alterations in serum composition. Table II includes a calculation of radiosodium exchangeability (ratio specific activity bone to serum) for rat bone of comparable water content, based on data previously reported(3). In the older animals only about a quarter of the exchangeable fraction can be classed as readily "available" to the extracellular fluid; the value for the weanlings is about two-thirds.

In the present experiments the observed changes in bone Na were limited to that portion contained in what we have termed the "fluid" phase. There was no evidence for alteration in "crystal" phase Na. The sodium of this latter phase must be tightly bound in some fashion to the bone solid. If it is the hydration shell about the crystals, the concentration must be exceedingly great, as the following calculation will show. For the 3 groups of animals, Na "space" averaged 90.7% of bone water. If all of the "crystal" phase Na is in the remaining water, its concentration would be 1,100, 4,700, and 10,400 meq/l. for the 20, 40 and 90 day animals, respectively. The latter value (61% saline) exceeds the solubility of NaCl in water by a factor of almost two. Values such as these could be used to support the hypothesis that "crystal" phase Na is indeed located on the crystal surface, as Neuman and Neuman have suggested(13).

It should be emphasized that our results pertain only to the effects of *acute* changes in extracellular fluid. In experiments of longer duration, inevitably involving some growth of bone (at least in the rat), changes in "crystal" phase Na might well occur. Such procedures as chronic acidosis, or adrenalectomy, might also produce effects on bone matrix.

In designing this experiment we deliberately set out to provoke extreme changes in serum Na concentration. With these data at hand, it is possible to predict the degree of change to be expected from any given degree of hypo- or hypernatremia. For example, a reduction of serum Na to 120 meq/l would cause a loss of 5.8% of bone Na in weanling animals, but only 1.5% in adults. The latter decrement is too small to detect, and this may account for the fact that some investigators are able to report small changes, and others no change. Most workers, in studying hyponatremia, have usually not lowered serum Na below this level.

Summary and Conclusions. Profound hypo- and hypernatremia were produced in rats of varying age. Resulting changes in bone Na content were much more pronounced in the younger animals, and decreased progressively as the animals became older. Calculated Na "space" of bone averaged 91% of bone water content for the 3 age groups studied. Observed changes in bone Na content could be ascribed entirely to that portion within the "fluid phase" of bone. There was no evidence of a change in "crystal phase" Na. Observed changes in adult rats were so slight that rather large changes in serum Na would be necessary for their detection.

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Skin Reactivity in Guinea Pigs Sensitized to Flea Bites: The Sequence of Reactions.* (27040)

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It is generally recognized that both humans and animals manifest various types of skin response to bites of hematophagous insects. Some individuals fail to react to bites, some have delayed or immediate skin reaction, while others show both immediate and delayed skin responses. Repeated exposure of an individual to bites of a given species ultimately may completely alter the type of reactivity(1-4,6). Mellanby(3), on the basis of limited clinical observations on humans has shown that, following repeated exposures over varying periods of time, allergic skin responses to mosquito bites are manifested by delayed skin reactions followed by the immediate type. He presented the hypothesis that the skin response of man to mosquito bites occurs in 4 distinct stages: 1) delayed, 2) delayed and immediate, 3) immediate only, 4) non-reactive. However, no experimental evidence was presented for the development of the state of non-reactivity. Generally, in hypersensitivity involving delayed skin reactions, little is known about hyposensitization or desensitization following repeated introduction of antigen. For example, equivocal success has been achieved in desensitization to tuberculin (8). On the other hand, depending on the desensitizing dose, desensitization of guinea pigs to protein antigens has been achieved for periods not exceeding 10 days(9).

The following study indicates that a definite sequence of types of reactivity occurs in guinea pigs in response to repeated exposure to flea bites, and that this sequence terminates in a stage of non-reactivity which lasts for at least 5 months.

Experimental. Three groups of guinea pigs (5 per group) were sensitized to flea bites by daily exposure of each animal to bites of 20 cat fleas, *Ctenocephalides felis felis* (Bouche) according to the method described by Benjamini, Feingold, and Kartman(6). When delayed reactions appeared approximately 5-6 days after the initial exposure, the animals were further exposed twice per week with few exceptions (Table 1) for about 14 weeks. One group was exposed to bites of 20 fleas per

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animal; a second group to bites of 200 fleas; and the third group was exposed once to bites of 20 fleas and once to bites of 200 fleas during the same week. Each animal was observed for skin reactivity at 20 minutes, 1 hour, 4 hours and 24 hours following the exposure. Immediate reactions were considered those which appeared within 4 hours; reactions which appeared after 18-24 hours were classified as delayed.

During the course of the work the number of animals used decreased due to traumatic effects of cardiac puncture and natural causes (the actual number of animals available for each challenge is shown in Table 1. Note that during the period of sensitization through July 23, the control animals are included in the figures).

Controls consisted of 4 additional groups of 5 animals per group which were sensitized to flea bites by daily exposure of each animal to 20 fleas. At the end of 5-7 days, these animals reacted with delayed skin reactions which appeared 18-24 hours following the bite. After 2-5 additional days of exposure to bites of 20 fleas, the animals reacted with both delayed and immediate skin reactions, the immediate skin reactivity appearing 30-60 minutes following the bite. At this point, exposures of the 4 control groups were stopped except for challenges with bites of 20 fleas per animal which were given as follows: one group at 3 months, one at 6 months, another at 12 months and the last group at 18 months following initial exposure.

Results and Discussion. Since no significant variation in reactivity occurred in the 3 groups of experimental animals (those which were exposed to 20, 200, or 20 and 200 fleas twice a week, respectively), the data from these groups were pooled.

The data presented in Table 1 clearly demonstrate the occurrence of a definite sequence in type of response of guinea pigs to bites of fleas. The characteristic lesions involved in delayed or immediate skin responses to flea bites have been previously described(6). During the first 3-4 days of exposure only a few animals reacted. Most animals showed delayed skin reactivity after 5 days of exposure, the reactions appearing 18-24 hours following the bite. In all, a period of 5-6 days was required for most animals for initiation of hypersensitivity. With continued exposure to the antigenic stimulus (flea bites) the animals responded with immediate reactions which developed into the delayed type. The immediate reactions appeared approximately 9 days following initial exposure (2-5 days after appearance of the delayed reactions) and became prominent within 20 minutes to 1 hour following the challenging bite. During a period of approximately 7 weeks, most animals exhibited both delayed and immediate skin reactions; however, the skin reactivity of some animals alternated between delayed only and delayed plus immediate. Toward the end of this period, the percentage of animals showing both types of reactions decreased, while the percentage of those with solely immediate reactivity increased sharply. Thus, the reactivity entered a new phase characterized by only immediate skin responses appearing within 20 minutes to 1 hour following the bite and completely fading within 4 hours. This phase lasted approximately one month, after which time the majority of animals became non-reactive.

In contrast to the test animals (see above) all control animals which were challenged at 3, 6, and 12 months following the initial exposure responded to the challenging bites with both immediate and delayed skin reactions. Of the 5 animals in the control group which were challenged 18 months following the initial exposure, 2 reacted with both immediate and delayed reactions, one showed delayed reactions only and the remaining 2 guinea pigs died before they could be tested. These results indicate that without repeated exposures, the hypersensitive state persisted at least 12-18 months.

The state of non-reactivity of the experimental animals was very likely the result of repeated exposure of the animals to the material introduced by the flea bites. With the establishment of a condition of relative desensitization it was of interest to determine the duration of this state. To test this, starting on Nov. 3, the guinea pigs were challenged periodically with bites of 20 fleas each on the dates shown in Table 1. It was found that the period of non-reactivity lasted for at least 5 months.

Summary. Experimental evidence is presented which demonstrates the occurrence of a

TABLE I. The Sequence of Skin Reactivity of Guinea Pigs to Flea Bites Following Repeated Exposures.

Reactivity Stage	Date of Exposure	No. of Animals Used	Delayed Reactions Only		Delayed and Immediate Reactions		Immediate Reactions Only	
			No. Reacting	% Reacting	No. Reacting	% Reacting	No. Reacting	% Reacting
Stage I Induction	July 16, 1960	30	2	6.7	0	.0	0	.0
	17	30	2	6.7	0	.0	0	.0
	18	30	3	10.0	1	3.3	0	.0
	19	30	8	26.6	4	3.3	0	.0
Stage II Predominantly Delayed	20	30	22	73.4	3	10.0	0	.0
	21	25	18	72.0	2	8.0	0	.0
	22	25	9	36.0	7	28.0	0	.0
	23	30	23	76.6	3	10.0	0	.0
Stage III Delayed and Immediate	24	14	0	.0	8	57.1	4	28.6
	25	14	6	42.8	6	42.8	2	14.3
	Aug. 1	14	9	64.2	5	35.7	0	.0
	8	12	2	16.7	4	33.3	4	33.3
	15	12	12	100.0	0	.0	0	.0
	19	12	1	8.3	11	91.7	0	.0
	22	12	8	66.6	0	.0	0	.0
	25	12	5	41.6	6	50.0	0	.0
	29	12	6	50.0	0	.0	0	.0
	Sept. 1	12	8	66.6	0	.0	0	.0
	6	12	1	8.3	3	25.0	1	8.3
	8	11	0	.0	5	45.5	4	36.4
	12	12	0	.0	4	33.4	0	.0
Stage IV Predominantly Immediate	16	12	1	8.3	1	8.3	4	33.3
	20	12	1	8.3	3	25.0	6	50.0
	22	12	0	.0	3	25.0	7	58.3
	26	12	0	.0	0	.0	10	83.4
	29	12	0	.0	0	.0	12	100.0
	Oct. 4	12	0	.0	0	.0	12	100.0
	6	12	0	.0	0	.0	12	100.0
	11	12	0	.0	0	.0	11	91.7
	13	12	0	.0	1	8.3	6	50.0
	17	12	0	.0	0	.0	5	41.7
Stage V Non-Reaction	21	11	0	.0	0	.0	0	.0
	27	11	1	9.1	0	.0	0	.0
	Nov. 3	11	0	.0	1	9.1	1	9.1
	10	11	0	.0	1	9.1	1	9.1
	17	11	0	.0	0	.0	4	36.4
	Dec. 1	10	0	.0	0	.0	0	.0
	Jan. 3, 1961	10	0	.0	0	.0	0	.0
	Feb. 1	10	0	.0	0	.0	0	.0
	April 17	9	0	.0	0	.0	0	.0

definite sequence of events in the skin reactions of guinea pigs following repeated exposure to flea bites. The sequence was: I—induction of sensitivity; II—predominately delayed skin reactivity; III—delayed and immediate responses; IV—predominately immediate reactivity; V—non-reactivity.

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Excretion of Formiminoglutamic Acid as an Index of Vitamin B_{12}, Folic Acid, and Methionine Deficiencies.*‡ (27041)

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Excretion of large amounts of formiminoglutamic acid (FGA), a metabolite of histidine, has been observed in Vit. B_{12} deficient rats(1) and chicks(2) fed diets borderline with respect to methionine requirement. In each species, supplemental methionine caused a reduction of FGA excretion to negligible levels, similar to those of the Vit. B_{12} supplemented animals. After a histidine load, high levels of FGA have been excreted by human beings deficient in folic acid(3-5), whereas patients with pernicious anemia failed to excrete significant amounts of FGA under the same conditions(3,4). However, Zalusky and Herbert(5) reported high excretion rates of FGA in 2 of 6 Vit. B_{12} deficient patients. In the present studies, methionine lowered excretion of FGA more effectively in chicks deficient in Vit. B_{12} than in chicks deficient in folic acid. Chicks that were moderately to severely deficient in methionine did not excrete significant amounts of FGA when folic acid and Vit. B_{12} were present in the diet.

Methods. Day old female New Hampshire chicks were distributed into groups of 6 to 8 each and placed in electrically heated batteries with screen wire floors. They received food and water *ad libitum.* Diet C62(6), which was fed in some experiments, was a purified diet that contained 30% of soybean protein,† 0.3% of L-cystine, and 24% of fat. When folic acid was omitted from Diet C62, the chicks grew almost as well as the controls. The soybean protein was found to be grossly contaminated with folic acid-active material. The following procedure was satisfactory for removing most of this activity. A 5% suspension of the protein in distilled water was held at pH 8 or slightly above for 5 minutes by addition of 6N NaOH. The protein was allowed to settle and the aqueous layer was siphoned off. The remaining liquid was removed by a cloth filter press; the protein was washed several times with distilled water and finally with ethanol. The protein was forced through a 14 mesh sieve, spread in thin layers on enameled trays, and dried in a forced air oven at 50°C for 2 hours. About 85% of the initial protein was recovered after the extraction process. Total folic acid activity, determined with *Lactobacillus casei,* was 375 mμg/g for one batch of starting protein and activity after extraction was 25 mμg/g. This specially extracted protein was satisfactory for producing severe folic acid deficiency in chicks and in the complete diet it was equal to the original protein in supporting normal growth. Omission of Vit. B_{12} from Diet C62 resulted in a severe deficiency which was the same with either the original or the extracted protein.

Two diets were used for producing a deficiency of methionine. One of these, Diet C69M, was exactly like Diet C62 except that L-cystine was omitted and 100 g of the soybean protein and 10 g of glucose in each kg of diet were replaced with 110 g of amino acid mixture 13,§ which simulated the composition of the soybean protein except methionine and cystine were omitted. Diet C68M contained (g/kg of diet): vitamin-free casein 100, gelatin 80, amino acid mix 5 (minus methionine) 115, L-arginine•HCl 8, corn oil 40, hydrogenated vegetable oil (Crisco) 200, Fox-Briggs Salts N 60 (7), choline chloride 2, crude glucose (Cerelose) 395, and vitamins at the same levels as

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* Part of these data were presented at the meeting of the Poultry Science Assn., Aug. 8-11, 1961, University Park, Pa.

‡ The authors wish to thank Mrs. Marjorie K. Romine for microbiological folic acid analyses, and Mrs. Esther M. Hurley and Mr. Woodrow W. Duvall for excellent assistance with these experiments.

† Cl Assay Protein purchased from Archer-Daniels-Midland Co., Cincinnati, Ohio.

§ Amino acid mix 13 (minus cystine and methionine) contained in g: DL-alanine 3.6, L-arginine•HCl 8.3, DL-aspartic acid 6.2, L-glutamic acid 19.3, glycine 4.1, L-histidine•HCl 2.6, DL-isoleucine 13.0, L-leucine 7.5, L-lysine•HCl 6.8, DL-phenylalanine 5.0, L-proline 2.5, DL-serine 6.9, DL-threonine, 7.8, L-tryptophane, 2.0, L-tyrosine 3.4, DL-valine 11.0.

TABLE I. Effects of Methionine Supplementation and Variation of Histidine Intake Upon Excretion of Formiminoglutamic Acid by Folic Acid Deficient, Vitamin B_{12} Deficient, and Control Chicks.*

Day	L-Histidine·HCl %	DL-Methionine %	Folic acid deficient Body wt g	Folic acid deficient FGA†	Vit B_{12} deficient Body wt g	Vit B_{12} deficient FGA†	Control Body wt g	Control FGA†
1	1	0	92	79	116	91	205	15
2	1	0	94	96	120	119	220	16
3	1	1	104	44	132	13	240	4
4	1	1	114	44	147	7	260	1
5	2	1	116	69	160	20	280	2
6	2	1	121	84	174	44	299	4
7	4	1	124	122	188	50	318	6
8	4	1	126	232	197	91	328	30

* Average of values from 2 experiments, 12 chicks/treatment, 22 days of age on day 1. Chicks were fed Diet C62, complete or deficient as indicated, prepared with specially extracted soybean protein.

† μmoles FGA/mole of histidine consumed/24 hr.

those in Diet C62. Amino acid mix 5(8) was formulated to simulate the amino acid composition of casein.

When the chicks were 3 to 4 weeks old, they were placed in individual cages. The procedures for collection of excreta and determination of FGA have been described(6). During each collection period, L-histidine•HCl was incorporated in the diet at a level of 1%, except in experiments presented in Table I, where higher levels were also fed.

Results. Data on FGA excretion of chicks deficient in folic acid and Vit. B_{12} are presented in Table I. With 1% L-histidine•HCl added to the diet (days 1 and 2), excretion of FGA was in the same elevated range for both deficient groups. The controls excreted much less. Addition of 1% DL-methionine to the diet caused a more pronounced drop in FGA excretion of Vit. B_{12} deficient chicks than that in the folic acid deficient group. The excretion of the control animals was also decreased. As the histidine supplement was increased to 2 and 4% of the diet, FGA excretion rose in both deficient groups and the amounts of FGA excreted were much higher for the folic acid deficient than for the Vit. B_{12} deficient chicks. Only on day 8 did the higher intake of histidine cause an increase in excretion of FGA by the control chicks. The daily food intake of the folic acid deficient chicks ranged from 7 to 12 g/100 g body weight, that for Vit. B_{12} deficient chicks from 9 to 12 g, and for the controls 7 to 11 g. Initial mean body weights of the 2 deficient groups were not too different; however, growth rate of the Vit. B_{12} deficient chicks was much greater than that of the folic acid deficient chicks, once the methionine supplement was added to the diet.

The effect of methionine deficiency upon FGA excretion is presented in Table II. Chicks fed either Diet C68M or C69M were severely

TABLE II. Excretion of Formiminoglutamic Acid by Methionine Deficient, Methionine-Vitamin B_{12} Deficient, and Control Chicks.*

Diet	DL-Methionine supplement %	Age, 3 wk No. chicks	Age, 3 wk Body wt g	Age, 3 wk FGA†	Age, 4 wk No. chicks	Age, 4 wk Body wt g	Age, 4 wk FGA†
C68M‡	0	—	—	—	16	107	1.2
“	.2	—	—	—	7	270	.9
“	.6	—	—	—	15	364	.2
C69M‡	0	15	84	14	8	93	16
“	.15	8	154	1.9	—	—	—
“	.7	14	214	.5	8	305	.2
C69M, No B_{12}	0	8	75	48	6	93	60

* When number of chicks exceeds 8, values are the average of 2 experiments. With Diet C69M, the data for 3 and 4 weeks are from chicks in different experiments.

† μmoles FGA/mmole histidine consumed/24 hr.

‡ By calculation Diet C68M contained (g/kg diet) cystine 1 and methionine 4; Diet C69M contained cystine 1.2 and methionine 2.

deficient in methionine as indicated by the very poor growth and feathering. Their long primary wing feathers curved upward near the ends and the upper wing covert feathers curled outward from the wing. With each diet the smaller methionine supplements resulted in intermediate rates of growth. Excretion of FGA was very low by all chicks fed Diet C68M irrespective of methionine supplementation. Chicks fed Diet C69M excreted small amounts of FGA; but with each level of methionine supplementation, FGA excretion was very low. When Vit. B_{12} was omitted from the methionine-low Diet C69M, growth was similar to that obtained with Diet C69M; however, the FGA excretion was markedly elevated by this omission of Vit. B_{12}.

When choline was omitted from Diet C62, the chicks grew slowly and developed perosis. Mean daily excretion of FGA was 6 and 8 μmoles FGA/mmole histidine/day for choline deficient chicks in 2 experiments.

Discussion. To a limited extent these data support the studies with human beings in which high FGA excretion was associated with folic acid deficiency but not with Vit. B_{12} deficiency(3,4). For example, when the chicks received 1% L-histidine•HCl and 1% DL-methionine supplements (days 3 and 4, Table I), FGA excretion of the Vit. B_{12} deficient chicks was almost as low as that of the controls, whereas, the folic acid deficient chicks excreted large amounts of FGA. On the other hand, subsequent elevation of dietary histidine in presence of the methionine supplement caused FGA excretion of the Vit. B_{12} deficient chicks to rise higher than that of the controls. Thus it would appear that the relative differences between histidine and methionine intakes may provide an explanation for the low FGA excretion reported for human beings (3,4) and the high values reported for chicks (2) and rats(1) deficient in Vit. B_{12}. It is difficult to compare the histidine loads received by the young growing chicks and the human beings (chiefly adults); however, the following calculations provide a very rough basis for comparison. In relation to body weight, total histidine intake of the chicks fed 1% L-histidine•HCl (approximately 1.5 g/kg body weight/day) was about 7 times that for a human being receiving a daily dose of 15 g L-histidine•HCl, as recommended by Luhby *et al.* (3), assuming a 65 kg man with a daily protein consumption of 65 g. In relation to protein intake under the same conditions, only 5.2% of the chicks' total protein plus free amino acid intake was histidine while the proportion of histidine would be around 18% for man. Presumably the folic acid and Vit. B_{12} deficient humans received adequate methionine. It would be of interest to study the effect of methionine-low diets upon FGA excretion in the Vit. B_{12} deficient patients. It is significant that increasing the L-histidine•HCl supplement of the Vit. B_{12} deficient chick to 2 and 4% of the diet caused appreciable excretion of FGA even in the presence of 1% methionine whereas the controls were able to metabolize these higher levels of histidine almost completely.

Unquestionably the severity of the folic acid and Vit. B_{12} deficiency states influences the excretion of FGA and the response to supplemental methionine. Silverman and Pitney(1) found that a 1% supplement of methionine caused greater reduction in FGA excretion by folic acid deficient rats than was observed in the present study with chicks; however, their deficient rats were growing at the same rate as the controls and presumably were less severely deficient than these chicks. Although the folic acid deficient and the Vit. B_{12} deficient chicks grew at similarly slow rates they cannot necessarily be regarded as being equally deficient. When the Vit. B_{12} deficient chicks received supplemental methionine, they were able to utilize the diet more effectively for growth. Their total daily food intake increased, but remained fairly constant in relation to body weight. The greater utilization of histidine for tissue protein synthesis probably did not affect the rate of FGA excretion since the requirement of histidine for the growing chick is 1.5 g/kg diet. With the 1% supplement of L-histidine•HCl, total histidine content of the diet was 15.9 g/kg and with the 4% supplement, 40.2 g/kg. Confirmation that growth rate did not affect FGA excretion has ben provided by a preliminary experiment in which food intake of the Vit. B_{12} deficient and control groups was limited to about ¾ of the expected intake beginning the day the methionine supplement was incorporated in the diet. The FGA excretion pattern of these chicks was similar to that of their corresponding groups in Table I (unpublished data).

The function of tetrahydrofolic acid in the metabolism of the formimino group of FGA has been delineated by Tabor and Wyngarden (9). No such role has been described for Vit. B_{12}. From the data in this paper it seems possible that Vit. B_{12} affects utilization of FGA in some way in addition to that involving the metabolism of methionine. The 1% level of methionine did not cause FGA excretion of the Vit. B_{12} deficient chicks to stay at the same low level as that of Vit. B_{12} supplemented chicks when histidine was fed at the highest levels. Only 0.3% supplemental methionine is required to support normal growth of B_{12} deficient chicks with this diet(10). Secondly, the chicks that were severely deficient in methionine did not excrete large amounts of FGA until Vit. B_{12} was omitted from the diet. Perhaps either methionine or Vit. B_{12} or both modify the cofactor role of folic acid in the metabolism of the formimino group by the chick in a manner like that demonstrated in the rat by Noronha and Silverman(11).

Summary. Day old chicks were fed a diet borderline in methionine for 3 to 4 weeks. They excreted large amounts of formiminoglutamic acid (FGA), a metabolite of histidine, when either folic acid or Vit. B_{12} was omitted from the diet. FGA was excreted in very small amounts by control chicks fed the complete diet. Incorporation of supplemental methionine at 1% of the diet caused a marked reduction in FGA excretion of Vit. B_{12} deficient chicks and a moderate reduction in the folic acid deficient chicks. Further elevation of dietary histidine and continuation of the supplemental methionine resulted in increased FGA excretion which was relatively greater in the folic acid deficient than in the Vit. B_{12} deficient group. Only on the last day of the experiment was the excretion elevated for the control group. Chicks fed diets severely deficient in methionine did not excrete large amounts of FGA until Vit. B_{12} was omitted.

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Inhibition of Aminonucleoside Nephrosis in Rats. II. Effect of Nucleic Acid Precursors and L-triiodothyronine.* (27042)

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The mechanism by which the aminonucleoside (P-A) of puromycin induces the classical nephrotic syndrome in rats is still unknown. Since adenine, a primary precursor of RNA in rat, has been shown partially to block the nephrotoxicity of P-A(1,2), it seemed worthwhile to evaluate the protective action of other RNA and DNA intermediates.

Methods. Female rats, descendants of the

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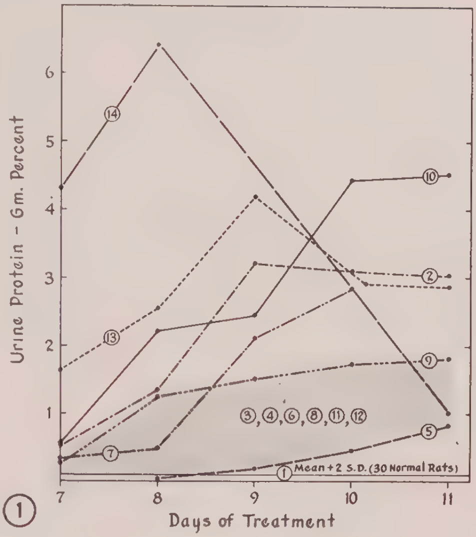
Holtzman strain, weighing 200±20 g were housed in individual metabolism cages in a temperature-controlled room (76° ±2°F) and maintained on Fox Chow and water *ad lib.* They were injected in the evening with an aqueous solution or suspension of P-A and nucleic acid intermediates as shown in Table I. Each was injected at a separate site subcutaneously, and P-A was always injected 15 to 30 minutes after the intermediate. In order to minimize sloughing at site of injection, the pH of each injected intermediate was adjusted to 6 to 8 with 1 N NaOH or phosphate buffer unless otherwise specified. The unsterilized preparations were kept frozen until they were ready for injection at which time they were thawed to a liquid state. The amount to be injected was removed and brought to body temperature, and the bulk of the material was immediately refrozen. Urine was collected daily beginning on the 7th day of injection and protein concentrations were determined by the method of Shevsky and Stafford as modified by McKay(3).

In a separate experiment 2.5 μg of L-triiodothyronine (T-3) was administered subcutaneously into 6 rats daily for 20 days. Beginning on the 8th day P-A was also administered as shown in Fig. 2.

Results. The details regarding the dosage schedule of the nucleic acid intermediates are listed in Table I and their effect on protein excretion is shown in Fig. 1. L-Glutamine (curve 13) and D-glucosamine (curve 14) appeared to cause earlier and more severe proteinuria than P-A alone (curve 2); however, this effect was not sustained. Uridine (curve 10) had no important influence initially, but protein excretion on the 10th and 11th days exceeded that of any other group. The remainder of the nucleic acid intermediates including guanine, adenylic acid, 4-amino-5-imidazole carboxamide, D-ribose, orotic acid, 2,6-diaminopurine, and thymine appeared to decrease the severity of the proteinuria and are shown grouped together in the shaded area of Fig. 1.

Rats treated with T-3 developed proteinuria 2 days earlier and excreted protein in much greater quantity than animals treated with P-A alone (Fig. 2).



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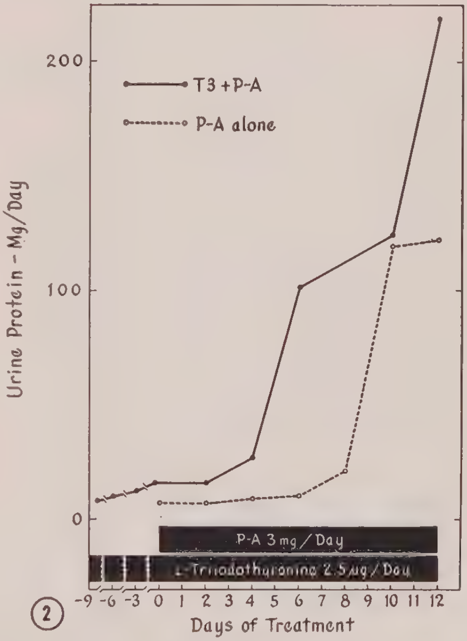

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FIG. 1. Effect of nucleic acid intermediates on P-A induced proteinuria. Numbers refer to compounds listed in Table I.

FIG. 2. Enhancement of P-A induced proteinuria by pretreatment with L-triiodothyronine.

Discussion. The demonstration that adenine afforded partial protection against P-A induced nephrosis(1,2) suggested that the nephrotoxic agent competed successfully with nucleic acid intermediates for polynucleotide or nucleoprotein synthesis. Glutamine contributes 3 nitrogen atoms to position 1, 3, and 9

TABLE I. Dose Schedule of Nucleic Acid Intermediates Administered with P-A.

Intermediate	No. of rats	I† concent. %	No. daily doses	Mole† ratio, I/P-A
1. Control	30	—	—	—
PURINES				
2. P-A*	16	.5	10	—
3. P-A + guanine HCl	4	2.0	10	7/1
4. P-A + disodium guanylate	4	7.0	10	8/1
5. P-A + 2,6-diaminopurine sulphate	4	1.0	10	4/1
6. P-A + inosine	4	2.0	10	5/1‡
7. P-A + adenylic acid	4	7.0	10	10/1
PYRIMIDINES				
8. P-A + orotic acid	4	1.0	10	10/1
9. P-A + thymine	4	1.0	14	10/1
10. P-A + uridine	4	2.0	14	5/1
OTHER				
11. P-A + 4-amino-5-imidazole carboxamide	4	2.0	10	12/1
12. P-A + ribose	4	2.0	10	13/1
13. P-A + glutamine	4	1.25	10	5/1
14. P-A + D-glucosamine	4	2.0	10	5/1

* Aminonucleoside standard dose = 1.5 mg/100 g body wt/day.
† I = Intermediate.
‡ 2.5/1 on day 3.

of the purine ring(4) and, therefore, this compound might be expected to accelerate the synthesis of adenine, which is the most important nucleic acid precursor in the rat(5). However, the fact that glutamine accentuated the proteinuria argues against such a mechanism. Based on the assumption that P-A might be incorporated into mucoprotein, a more likely explanation for the unfavorable effect of both glutamine and glucosamine is the fact that hyaluronic acid which is present in cellular basement membrane is synthesized from glucosamine, which in turn derives its amino group from glutamine(6,7). It is interesting that glucosamine and P-A are aminosugar and aminosugar derivative, respectively. Replacement of the aminosugar moiety in P-A with a normal sugar (D-ribose) has been shown to prevent its nephrotoxic action(8). Except for uridine, the other nucleic acid intermediates (i.e. thymine, guanine, adenylic acid, 4-amino-5-imidazole carboxamide, D-ribose, orotic acid, 2,6-diaminopurine, and inosine) reduced the toxicity of P-A.

Thyroxine has been shown to result in uncoupling of oxidative phosphorylation(9) and in swelling of rat kidney mitochondria accompanied by increased oxidation of DPNH(10). These effects, which may be related, are believed to be secondary to increased permeability of mitochondrial membrane to substrate. ATP can prevent swelling caused by thyroxine(11). The kidney is also uniquely different from other organs in having a very high rate of oxygen consumption(12) and the ability to convert thyroxine to triiodothyronine(13). It is not surprising, therefore, that the presumed increased metabolic rate following T-3 administration was accompanied by early and more severe proteinuria. The accumulation of interstitial fluid rich in mucopolysaccharide which occurs in absence of thyroxine has been pointed out by Watson and Pearce(14) and by Ludwig et al(15). The mechanisms of action of thyroid hormones have been thoroughly discussed recently(16).

Summary. The aminonucleoside of puromycin (P-A), which produces the nephrotic syndrome in rats, was administered simultaneously with various nucleic acid intermediates. L-glutamine, D-glucosamine, and uridine intensified the nephrotoxicity, whereas guanine, adenylic acid, 4-amino-5-imidazole carboxamide, D-ribose, orotic acid, 2,6-diaminopurine, inosine, and thymine appeared to ameliorate the condition. Rats primed with L-triiodothyronine (T-3) developed proteinuria earlier than rats treated with P-A alone.

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Mechanism of Action of Staphylococcal Toxin in Rabbits.* (27043)

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Introduction. Bacterial exotoxins are known to inflict serious damage on specific systems. Thus, the alpha toxin of the Welch bacillus contains a red-cell lecithinase; the diphtheria toxin produces a myocardial lesion; and the exotoxin of the Staphylococcus when given intravenously causes renal necrosis. Bacterial exotoxins, like endotoxins, also can cause peripheral vascular collapse. The endotoxins do so when the reticulo-endothelial system (R.E.S.) loses its capacity to destroy them, so that they remain free in the circulation to inflict vascular damage(1). If exotoxins can injure the R.E. system, the shock and death from exotoxins might be brought about by development of a secondary endotoxemia, the source of which are the endotoxins continuously entering the circulation from the intestine(2).

Some of the experiments to be reported below were done to see whether the death caused by staphylococcal toxin is due to loss of R.E.S. function. Other experiments were done to see whether the renal injury which is a constant feature of the pathology at death from this exotoxin is involved in the death.

Materials & Methods. Adult albino rabbits (wt 2kg) were used throughout. Two separate batches of staphylococcal toxin† were used. The MLD/100 of a 1:10 dilution in saline was determined by administering increasing volumes into the ear veins of rabbits until the amount necessary to kill all rabbits within 24 hours was reached. For the first batch of toxin this MLD/100 was 1.6ml/kg, and for the second batch 2.5ml/kg. Gross postmortem examination was performed, but only the kidney was studied histologically‡.

To exclude contamination of the exotoxin with endotoxin each batch of exotoxin was extracted by the Boivin method(6), and found to contain no endotoxin on assay by the chick embryo technic(7).

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† Staphylococcal toxin, #42925-199 (Wood #46) preserved in 0.01% thiomerosal, Lederle Lab., Pearl River, N. Y.

‡ The effect of intravenously administered Staphylococcal toxin on the kidney is well established(3,4,5) and consists of diffuse cortical necrosis and focal medullary hemorrhagic necrosis. Other findings at death in about ½ the animals in a series include scattered petechial hemorrhages among the viscera.

The relationship between the R.E. system and the staphylococcal exotoxin was examined in 2 ways: (a) by testing the blood of the rabbit for endotoxemia following a lethal dose of exotoxin; and (b) by determining any change in toxicity of staphylococcal exotoxin in the rabbit with an injured R.E. system. Injury to the R.E.S. was inflicted by a non-lethal dose of Thorotrast (2.75ml/kg) given 3 hours before or by exposure of the rabbits to 2 hours of hemorrhagic shock(8,9) and assaying for toxicity 20 minutes after a curative transfusion.

The role of the renal lesion in the lethality of staphylococcal exotoxin was determined by noting survival time of rabbits nephrectomized 3 hours before a sublethal dose of exotoxin or 3, 7, or 10 hours after a lethal dose. This survival time was compared to that of rabbits to which no toxin was administered, but from which the kidneys were removed or the arterial circulation totally occluded. In the latter 2 groups of rabbits, survival time was 5 days.

The role of the vegetative nervous system in production of the renal lesion was studied by giving a sublethal dose of the exotoxin to rabbits with one renal pedicle denervated a week in advance. The effect of Dibenamine on the renal lesion was also observed(10). Survivors for examination were killed 48-96 hours later.

Results. Of 7 rabbits given a lethal dose of staphylococcal exotoxin none showed an endotoxemia. Pretreatment with Thorotrast did not alter the lethality of the exotoxin, but 2 hours of hemorrhagic shock increased it considerably (Table 1).

The usual survival time after an MLD/100 of toxin was 24 hours; after excision of both kidneys, 5 days. Removal of both kidneys 3 hours *after* a lethal dose of toxin delayed death until the fifth day in half the animals. Removal of both kidneys 7 or 10 hours *after* a lethal dose of toxin delayed death in all animals until the fifth day (Table 2).

TABLE I. Effect of Injury to Reticulo-Endothelial System on Mortality of Staphylococcal Toxin.

Dose of Toxin* ml/kg	No Injury	Thorotrast	Reversible Shock
.5	0/4	0/4	0/6
.8	0/4	1/4	2/4
1.0	0/6	0/6	4/6
1.2	3/13	5/13	2/2
1.4	4/5	3/5	—
1.6	9/10	4/4	—

* Toxin in 1/10 dilution.

Removal of both kidneys 3 hours *before* a sublethal dose of toxin reduced survival time from 24 hours to 2 hours in 1/2 the rabbits, and a uniformly non-lethal dose in normal rabbits reduced survival rate from 100% to 50% (Table 2).

Sympathectomy of a renal pedicle did not prevent renal necrosis. Indeed the injury was clearly worse on the denervated side. Dibenamine did not change survival time or renal pathology.

Discussion. These data indicate that the staphylococcal exotoxin does not affect the function of the R.E.S. or evoke an endotoxemia. Nor does the R.E.S. affect the mechanism of action of this toxin, since Thorotrast does not alter the lethal dose. On the other hand a brief exposure to hemorrhagic shock increases sensitivity to the exotoxin. This may be attributable to an adverse effect of shock on excretion of the toxin or on a detoxifying mechanism outside the R.E.S. Renal denerva-

TABLE II. Mortality of Staphylococcal Toxin in Nephrectomized Rabbits.

	Bilateral Nephrectomy No Toxin	Bilateral Renal Artery Ligation No Toxin	Bilateral Nephrectomy MLD/100 Toxin*		Bilateral Nephrectomy	
					Sublethal Dose Toxin†	MSD Toxin‡
			3 Hr Before	7-10 Hr Before	3 Hr After	
No. Exp.	10	5	8	10	10	6
Mortality 24 hr	0/10	0/5	4/8	0/10	10/10	3/6
5 days	10/10	5/5	8/8	10/10	—	6/6

* Minimum Lethal Dose (MLD/100) = 2.5ml/kg (1/10 dilution).
† Sublethal Dose = 2.0 − 1.8ml/kg (1/10 dilution).
‡ Maximum Survival Dose (MSD) = 1.4ml/kg (1/10 dilution).

tion, which blocks the effect of endotoxin on the renal cortex in the Shwartzman reaction(11), does not prevent the renal lesion caused by exotoxin. Thus it appears that the kidneys remove a considerable amount of staphylococcal toxin from the circulation, for bilateral nephrectomy performed 7 to 10 hours after the toxin is given prevents death. The same conclusion follows from the observation that death from toxin is quicker if the kidneys are removed before the toxin is given. Hence it appears that in the intact animal, extraction of toxin by the kidneys delays but does not prevent death. One may conclude further that while the toxin is held by the kidneys, it is not acting elsewhere. But sometime afterward the toxin or a product of the toxin interacting with the kidney is released and that this released material kills. Since ischemic necrosis of both kidneys does not kill earlier than bilateral nephrectomy, the necrosis produced by toxin is not the cause of death. Presumably, then, the toxin or a modification of it acts on an unidentified site elsewhere to cause death. Vasospasm and other effects of the catechol amines appear not to be involved. The mechanism of death following administration of staphylococcal exotoxin remains unknown.

Summary. The R.E.S., which is the main defense against endotoxin, plays no part in the defense against staphylococcal toxin. The kidneys remove staphylococcal toxin from the circulating blood. The toxin or a modification of it is subsequently released, then exerts a lethal action elsewhere. For some hours after it is taken up by the kidney the toxin cannot act. Thus, the kidney is protective for some hours. Indeed nephrectomy prevents death from exotoxin. The renal necrosis produced by the toxin in itself is not the lethal lesion, because renal necrosis produced by arterial occlusion does not cause such rapid death as occurs in renal necrosis produced by the toxin.

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Renin and Angiotensinase Content of the Kidney of Normal and Renal Hypertensive Rats.*‡ (27044)

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The possible role of renin as a causative agent in experimental renal hypertension has been extensively investigated. Direct determination of the concentration of renin in the blood has been indecisive. It is to be expected that if renin, through its pressor effect, is the direct cause of experimental renal hypertension, it would be present in the blood of hyper-

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tensives in abnormally high concentrations. Actually its concentration in both normal and hypertensive conditions is so low that no agreement has been reached by these studies(1).

Assay of renin in the kidney, where it is present in higher concentrations than in the blood, has been extensive but the results of these studies have not shown sufficient uniformity to permit a clear picture of what happens to the renin content of this organ as hypertension develops(2).

The current study has 2 objectives: (1) to make further observations bearing on renin content of the kidneys in experimental hypertension, and (2) to make simultaneous determinations of renal angiotensinase activity. Since the pressor response to renin is mediated through production of angiotensin, and since this polypeptide is rapidly inactivated by angiotensinase in blood and tissues, both the physiologic effect and the assayed activity of renin are dependent on the rate of this enzymatic inactivation of angiotensin. Variations in angiotensinase activity in hypertension conceivably could alter the pressor activity of renin and could interfere with the biological assay of its content in the kidney.

Methods. Albino rats of the Sprague-Dawley strain, weighing between 190 and 340 g, were used in all experiments. Hypertension was produced by partial occlusion of the left renal artery by means of a silver clip, as described by Schaffenburg *et al*(3). Blood pressures were measured by the Friedman and Freed tail microphone technique(4).

Rats were kept under observation for about 6 weeks (range 1-2 months) following operation. During this time blood pressure measurements were made at weekly intervals. When an animal was ready for testing it was killed by a blow on the head, the kidneys were removed and homogenized, individually, in 10 ml of saline per gram of tissue. The homogenate was centrifuged and the supernatant used for determination of renin and angiotensinase.

Two bioassay procedures were used for determination of renal renin concentration: (1) Direct Method. Six-tenths ml of kidney homogenate were administered intravenously to an assay rat. This animal was anesthetized with urethane (1-1.5 g/kg intraperitoneally) and blocked with pentolinium (3 mg/kg subcutaneously). Its arterial pressure was recorded continuously from a float mounted on a mercury manometer which was connected to the rat's femoral artery by means of a polyethylene cannula. The renin content of the homogenate was determined by referring the pressor response it produced to a dose-response curve constructed from the pressor responses recorded when a standard renin preparation was studied in a series of 38 rats. Each assay rat was used for only one renin determination, thus avoiding any effects of tachyphylaxis. Renin content was expressed in Goldblatt Units.

(2) Indirect Method. This method is based on production of angiotensin on incubation of renin with substrate *in vitro*. The angiotensin formed in presence of sufficient substrate and in absence of angiotensinase, is proportional to the amount of renin in the original sample. Angiotensinase was inactivated, with minimum destruction of renin, by acidifying with phosphoric acid to pH 2.0 for 60 min at 24°, then returning the mixture to pH 6.5 with sodium hydroxide. The angiotensinase-free mixture was then incubated for 2 hours with excess angiotensinogen prepared from normal dog plasma, according to the methods of Fasciolo and Taquini(5). This reaction was stopped by placing the sample in boiling water for 3 min. Three-tenths ml of the supernatant solution were injected into an assay rat prepared as described previously. A standard hog renin preparation** was treated in a similar way to produce angiotensin *in vitro*. From a comparison of the pressor response of the standard with those of the test preparations the concentration of renin in the kidney extract was calculated, in terms of Goldblatt Units.

For determination of angiotensinase the kidney homogenate was incubated with synthetic valine-5 angiotensin II amide*** (specific activity: 3 Goldblatt Units/μg) for 2 hours at 37°, then injected into an assay animal.

Individual kidneys from 3 groups of rats were studied: Group 1, normal, control rats; (average arterial pressure 111 mm Hg), 7

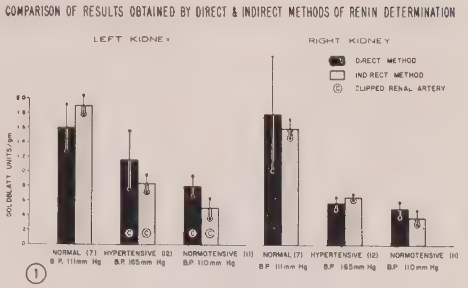
** We are indebted to Dr. Oscar Helmer, Eli Lilly and Co. for hog renin used.

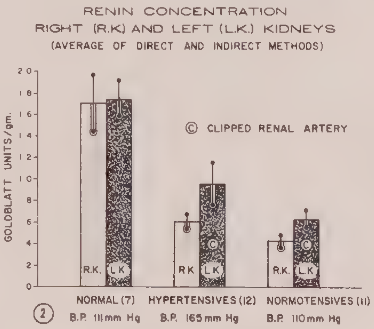
*** Obtained from CIBA Laboratories, Basel, Switz.

animals; Group 2, rats which developed hypertension following unilateral partial renal ischemia, (blood pressure 150 mm Hg or above), 12 animals; Group 3, rats which failed to develop hypertension after the procedure, (blood pressure less than 150 mm Hg), 11 animals. Rats of Group 3 will be referred to as "normotensives".

Results. The renin concentration as determined by the 2 methods (direct and indirect) was in satisfactory agreement (Fig. 1). This agreement was used as justification for averaging the values obtained by the 2 methods; these averages are used in the graph of Fig. 2. It is apparent that: (1) There is no difference between right and left kidneys of normal animals with respect to renin concentration. (2) Concentration of renin in the kidneys of animals subjected to partial occlusion of the renal artery was lower than that in the kidneys of control animals. This was independent of blood pressure elevation. The difference between kidneys of normal animals and kidneys of hypertensive animals with (P value <.025) or without (P value <.005) partial occlusion of the renal artery was statistically significant as also was the difference between kidneys of normal animals and kidneys of normotensive animals with (P value <.0005) or without (P value <.0005) partial occlusion of the renal artery. (3) Mean renin concentration was higher in the ischemic than in the non-ischemic kidneys. This was not dependent on blood pressure elevation. The difference was not statistically significant (P value >.1), possibly due to the small number of observations.

The data concerning concentration of angiotensinase in the kidneys of the several groups of rats (Fig. 3) show that: (1) There is no difference between right and left kidneys of normal animals in this respect. (2) Concentration of angiotensinase in kidneys of experimental animals which became hypertensive was equal to that in control animals. (3) Concentration of angiotensinase in kidneys of experimental animals which did not become hypertensive was less than in hypertensive or control groups. The difference was not statistically significant (P value >.1). Normotensive rats with contralateral nephrectomy (not represented in Figure) did not show this decreased angiotensinase concentration (this angiotensinase activity was 1153 μg of synthetic angiotensin inactivated/g, SE ± 164). (4) There was no difference in angiotensinase concentration between kidneys with arterial supplies partially occluded and those non-





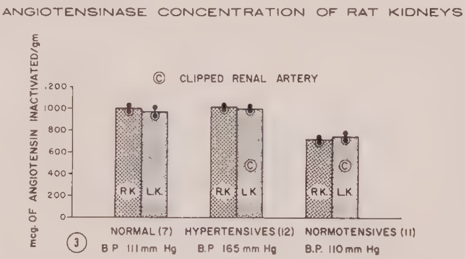


FIG. 1. Mean values (vertical bars) for renin concentrations for left and right kidneys of normal, hypertensive and "normotensive" rats. Vertical lines at top of bars represent standard error of mean. B.P. in each case is average mean blood pressure of the group. Numbers in parentheses are numbers of kidneys studied.

FIG. 2. Average mean values for renin concentrations in left and right kidneys of normal, hypertensive and "normotensive" animals. Vertical line at top of each bar represents standard error of mean. B.P. in each case is average mean blood pressure of group; in parentheses, number of animals in group.

FIG. 3. Mean values for angiotensinase concentrations for right (RK) and left (LK) kidneys of normal, hypertensive and "normotensive" rats. Standard error of mean is indicated as in previous figures. B.P. in each case is average mean blood pressure of each group; in parentheses, number of animals in group.

occluded. This was true whether or not the blood pressure became elevated.

Discussion. If the assumption that renin secretion parallels renin content in the kidney is correct, one is forced to conclude from our data that experimental renal hypertension in the rat is not caused by increased renin secretion; kidneys of renal hypertensive rats were consistently lower in renin concentration than kidneys of control rats.

This is in agreement with Beckwith and Chanutin(6) and Williams *et al*(7), who, working in the same species, found decreased pressor properties of kidney extracts in renal hypertension. Gross *et al*(8) and Tobian *et al*(9) on the other hand, found that the decrease in renin concentration was present only in the "untouched" kidney, while the ischemic or "clipped" kidney had a normal renin concentration.

Gross *et al*(8) and Tobian *et al*(9) found a higher concentration of renin in the ischemic kidney than in the contralateral. This is in accord with our results although we did not find the difference to have statistical significance, possibly due to the number of observations. The latter author has suggested that the decreased concentration is associated with increased stretch of the juxtaglomerular cells (the proposed source of renin) caused by increased arterial pressure(10). This interpretation is not in accord with the present data where the "untouched" kidneys of the experimental groups of rats had a similar renin concentration whether the pressure was elevated or not. The stimulus for regulating renin secretion remains undiscovered; it is unlikely that it is a circulating substance since after unilateral "clipping" there are differences in concentration between the 2 kidneys though they are both circulated by the same blood. Increasing amounts of evidence suggest that Na metabolism is closely related to renin and we are inclined to believe that this line of research is likely to disclose the stimulus for regulating the secretion of this enzyme.

Agreement was found between the direct and indirect methods for renin determination, making it unlikely that differences in these assay techniques account for the conflicting results in the literature.

Our data show that the angiotensinase concentration of kidneys of animals with renal hypertension remains in the normal range. Haynes and Dexter(11) found normal values for angiotensinase in plasma of patients with hypertension. Quinby *et al*(12) observed no difference in concentration of angiotensinase in renal arterial and venous blood of humans before and after complete occlusion of the renal artery for periods of 10 to 12 min. Weinstein *et al*(13) observed a diminution of angiotensinase in the venous blood from acutely ischemic kidneys. This observation was not confirmed by Braun-Menendez *et al*(1).

Our findings indicate that the concentration of angiotensinase is independent of renin content of the kidney. This is not surprising since it has been shown by Helmer *et al*(14) that the so-called "angiotensinase" represents a group of different substances which have the common action of inactivating angiotensin. In any event the observed differences in renin content of the kidney cannot be attributed to variations in their angiotensinase concentration.

Summary. Kidney renin and angiotensinase concentrations were determined for the following groups of rats: normals(7); rats with renal hypertension produced by unilateral clipping of the renal artery(12); and rats unilaterally clipped which failed to develop hypertension(11). Kidney extracts were assayed for renin by 2 methods: 1) direct intravenous injection into a rat sensitized by ganglionic blockade, and 2) *in vitro* production of angiotensin which was subsequently assayed in a similar manner. Angiotensinase concentration was determined by incubation of the kidney extract for 2 hours at 37° with a known amount of synthetic angiotensin and subsequent assay of the residual angiotensin. Renin concentration was reduced in kidneys of both groups of rats with renal artery clips. The reduction in the untouched kidney was greater than that in the clipped kidney. Concentration of angiotensinase in the kidneys of experimental animals which became hypertensive was equal to that in control animals.

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Studies of Congenital Transmission of a Leukemia Virus in Mice.* (27045)

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Gross reported in 1951(1,2) and in more detail later(3-5) that some of the extracts and filtrates prepared from leukemic tissues of AK strain mice induced lymphocytic leukemia in certain recipient mice, particularly in the C3H/Bi and C3Hf/Bi strains. Experiments were described(2, 6, 7, 8) of an attempt to study the "natural" transmission of the Gross virus in C3H mice. The concept was developed that the virus isolated from AK leukemic mice was responsible for the high frequency of lymphocytic neoplasms within that strain, and that the virus was transmitted through the germ lines resulting in successive generations of leukemic AK mice with similar frequencies and latent periods (See 7). It had to be assumed also, but this was not discussed in considering the concept, that the paternal line was as effective a means of transmission of virus as the maternal line(9). The results of the early "natural" transmission studies were equivocal(2). The frequencies of leukemia among the uninoculated offspring from virus-inoculated parents were suggestive of transmission of virus in some matings(7) but not in others, the latent period was long and too few mice were observed beyond one generation.

Recently, Gross has reported on certain aspects of the so-called "vertical" transmission of the Gross virus using a more highly potent, serially passaged virus(10).

All C3Hf/Bi offspring born to virus-inoculated females developed leukemia and average age at leukemic death was similar to that of the inoculated parents. None of the offspring born to non-inoculated females mated with virus-inoculated males had developed leukemia up to 9 months of age. The experiments were not designed to determine the manner of transmission of virus through the maternal line.

The isolation and concentration of a leukemogenic virus from transplantable Sarcoma 37(11,12) presented the opportunity to study certain epidemiologic aspects of this neoplastic disease in mice, including that of natural transmission of the virus. This report presents the results, preliminary in some aspects, of the fate of offspring in successive generations, born to parenterally-infected parents.

Materials and methods. Virus. The preparation designated LT(V) 96 used throughout these studies was prepared as follows: A pool of leukemic lymph nodes and spleens from

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* The technical assistance of Mr. Britton Smith is gratefully acknowledged.

virus-inoculated BALB/cAn mice served as source material. The tissues were extracted and the particulate fraction concentrated by differential centrifugation. The details of the operative procedures have been published(11). Final concentration of the virus material was 0.5 g equivalents per milliliter. A 1.0 g equiv. per ml concentrate is equal to 1 ml of final suspension for each gram of pooled tissue processed. Aliquots of the virus preparation were quickly frozen and stored at the temperature of a dry ice chest until used.

The mice which ranged in age from less than 1 day to 21 days were inoculated subcutaneously with 0.1 ml of a 10^{-1} dilution of the virus. The diluent employed was 0.05 M,pH 6.8, sodium citrate buffer containing 1.0 mg of crystalline hyaluronidase per 100 ml of medium. Exact times of inoculation are given in the individual experiments. All members of a litter were inoculated and were returned to their mothers where they remained until weaning at 4 weeks of age. Successive generations of uninoculated mice (G_1, G_2, etc.) were usually caged 3 females to 1 male.

Successive generations of control mice were maintained at the same time and were derived from non-inoculated litter mates of the mothers supplying the experimental mice.

The results of a bioassay of this virus preparation in newborn BALB/c mice, the standard reference strain, are presented in Table 1 for comparative purposes.

TABLE I. Bioassay of virus concentrate in newborn BALB/c mice.

Log_{10} Dilution	No. leukemic/ No. inoculated	Incidence of leukemia (%)	Avg Age at leukemic death (Mo)
−1	14/14	100	3
−2	18/18	100	4
−3	17/17	100	4
−4	5/5	100	4½

Mice. Four inbred strains of mice were used, principally, in this study: C3Hf/Lw, C3Hf/Gs, C3Hf/Fg, and RFM. All C3H strains were foster-nursed following removal by Caesarean section and did not carry the Bittner milk agent. The origin and characteristics of the C3Hf/Lw strain have been described(9,13). C3Hf/Gs mice were received from Dr. Ludwik Gross, through Dr. Ray Bryan of this Institute. Brother x sister inbreeding was continued from a single pair and pedigreed mice from our F_6 to F_8 were used. Spontaneously occurring neoplasms of reticular tissues are not common in either strain. A frequency of approximately 10% has been observed in our laboratory but the leukemias appear usually beyond 1 year of age. Although some are derived from cells of the lymphoid series, the thymus appears to play no part in the genesis of these. The C3Hf/Fg strain is a high-leukemic strain and the origin and characteristics of this strain have been described(9,14). A high frequency of different morphologic forms of leukemia has been observed throughout our F_1 to F_{14} breeding generations. The mean age at death from leukemia is approximately 14 months and rarely are the neoplasms observed in females before 7 months and in males before 9 months. Numerous attempts to isolate and concentrate a cell-free material from leukemic tissues of C3Hf/Fg mice have been unsuccessful(9).

RFM strain mice were originally obtained from the Jackson Memorial Laboratory in 1959 and inbreeding has been continued since that time. An occasional lymphocytic or granulocytic neoplasm has been observed in our colony but the strain is a low-leukemic strain. The incidence and latent period of the disease for RFM mice have been published by Upton and colleagues(15).

Results. The response of several strains of mice, not heretofore studied, to virus preparation LT(V) 96 is shown in Table II. A low frequency in C57BL mice observed previously(12) was observed here in another subline, C57BL/Ka. In addition, high leukemic C58 mice, related in origin to C57(16), and NH mice, particularly the latter, indicate a degree of resistance to the Moloney virus. The mean age at leukemic death also indicates a lower susceptibility in the Lw subline of C3H compared with the other 2 C3H sublines studied. It is clear under the conditions employed here that all of the strains studied show a lower susceptibility than BALB/c mice, the standard reference strain (Table 1).

Transmission Studies in C3Hf/Gs Strain Mice (Maternal Line Series N10). The untreated offspring of 2 virus-inoculated C3Hf/Gs litters (N10 and N13) have been observed

TABLE II. Induction of Leukemia in Inbred Strains of Mice by Moloney Virus Preparation LT(V)96.

Strain of Mouse	No. Litters Inoculated	Time of Virus Inoculation (days)	No. Leukemic / No. Mice	Age at Leukemic Death (Mo)	Transmission to Offspring via Mother[1]
C3Hf/Lw	2	7, 7	4, 4 – – 4 4	7, 7½	+
C3Hf/Gs	3	13, 8, 1	4, 3, 5 – – – 5 3 5	5, 3½, 4½	+
C3Hf/Fg	5	5, <1, 2, 14, 7	2, 7, 4, 0, 3 – – – – – 2 7 4 7 4	6, 3½, 5½, –, 4½	+
RFM	4	7, <1, 4, 1	3, 1, 3, 2 – – – – 3 1 3 3	4, 5, 4½, 3½	–
C58	4	6, 2, 5, 5	0, 1, 1, 0 – – – – 4 5 9 5	–, 5½, 4, –	–
C57BL/Ka	3	5, 10, 1	0, 1, 1 – – – 9 6 6	–, 4½, 6	?
AKR/Lw	4	20, 4, 5, 7	0, 7, 3, 2 – – – – 6 7 3 2	–, 4, 4, 5	?
NH	5	5, 21, 11, 2, 6	0, 0, 0, 0, 0 – – – – – 4 4 9 3 5	—	–

[1] At least one subsequent generation, including several litters, observed for each strain.

over a period of several successive generations. In the N-10 series a litter of 5 mice born to female 45820 received virus through a subcutaneous inoculation at 13 days of age. Two females developed leukemia at 3 and 3½ months of age and 2 of 3 males at 6 and 7 months of age. A litter of 3 was obtained from one of the virus-inoculated females and a litter of 5 from the second female, both mated with the virus-inoculated male which eventually developed leukemia at 6 months of age. All 8 untreated offspring (5 females and 3 males) born to the 2 inoculated females developed leukemia. Age at leukemic deaths ranged from 3½ to 7½ months and was similar to that for the virus-inoculated parents. Fig. 1 gives a detailed representation of the fate of the original virus-inoculated litter (G_0) and subsequent generations of untreated C3Hf/Gs mice born to infected[†] (but untreated) mothers. It may be seen that the majority of offspring developed leukemia in the G_2 generation, 5 litters from 5 different untreated G_1 females and in the G_3 generation, 5 litters from 5 different untreated G_2 females. Average age at leukemic death has remained essentially the same throughout the 4 generations. Three litters in the G_1 to G_3 series suckled mothers which became leukemic during the suckling period and 2 litters were born to the uninoculated (but infected) mothers mated with non-inoculated ("normal") C3Hf/Gs fathers. The frequency and latent period of the disease was essentially similar in these mice to others in the series.

Of interest is the observation that none of 4 mice of a second litter born to an infected female of the G_1 generation has developed leukemia in 10 months following removal by Caesarean section and foster nursing on a C3Hf/Lw mother. A subsequent generation of 6 mice also remains leukemia-free. All 4 of

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[†] The term "infected" will be used, for convenience, to mean those mice obtaining virus through their mothers. Virus-inoculated litters are designated G_0 and successive litters, obtained by matings of sibs, as G_1, G_2, etc. "Normal" refers to those mice having no apparent contact with virus.

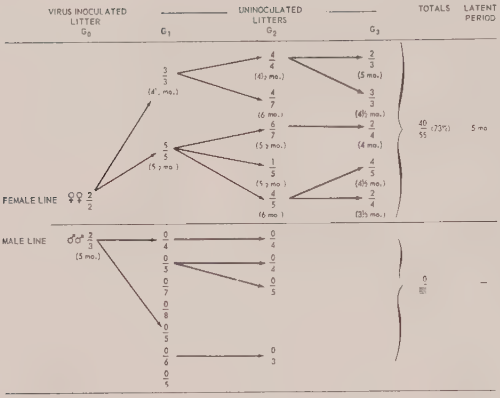

FIG. 1. Litter of 5 (2 females, 3 males) C3Hf/Gs mice, born to female 45899 (F90+6) inbred generation, inoculated at 13 days of age with 0.1 ml preparation LT(V)96 subcutaneously, (G_0). The 2 females were autopsied leukemic at 3 and 3½ months of age. Two of the males were leukemic at 6 and 7 months of age. Single litters (G_1) were obtained from each of the 2 potentially leukemic females No. 45925 and 45926, mated with one of the potentially leukemic males No. 45929. All subsequent litters in the female line were obtained by matings of potentially leukemic females with their potentially leukemic male litter mates (Female line). To establish the male line, potentially leukemic males (G_0) No. 45928 and 45929 were mated at 6 weeks of age with uninoculated C3Hf/Gs females of the same inbred family. Eight G_1 litters were produced as shown and these produced the 4 G_2 litters as shown. All of these offspring are now 8 to 10 months of age. Numbers above line refer to leukemic mice; below line, to total no. of mice in litter surviving to appearance of first leukemics within each litter. Latent period is average age at leukemic death in months.

the first litter which suckled their own mother became leukemic. A reciprocal foster-nursing of a litter of 6 C3Hf/Lw mice was accomplished using an infected C3Hf/Gs mother of the G_2 generation. Four of 6 in this litter have developed leukemia at 5 months of age.

Paternal Line. Each of the 2 virus-inoculated males of the N-10 series was mated with 3 uninoculated ("normal") C3Hf/Gs females of the same subfamily. Three litters (G_1) were fathered by the inoculated male which became leukemic at 5 months and 4 litters by the inoculated male which became leukemic at 7 months. Four G_2 litters were born to 4 different G_1 mothers mated with their litter mates. No leukemias have appeared among the 56 offspring obtained, now 8 to 10 months of age.

Series N-13. Three of 4 *(Series N-13)* C3Hf/Gs mice survived following inoculation of virus preparation LT(V) 96 at 8 days of age. These were born to female 45819 a litter mate to female 45820. All 3 (G_0), a female and 2 males developed leukemia at 3½, 3½ and 4 months.

A first litter of 3 females was born to virus-inoculated female mated to one of the inoculated males. All 3 (G_1) females, which were uninoculated, developed leukemia at 3½, 4 and 7 months. A litter was obtained from each of these 3 females (prior to developing leukemia), mated with a male from the same subfamily but having no contact with virus. The fate of the members of these 3 litters (G_2) was as follows: 2 out of a litter of 4, 5 of 5 and 3 of 4 died leukemic at 3½ to 7 months of age, average age at leukemic death being 5 months. A later generation (G_3) was obtained from one of the infected (but untreated) females of a G_2 litter. This female died from leukemia at 3½ months of age. Four of 6 of her litter have died leukemic at 3½ to 5 months. The controls for the N-13 series consisted of successive generations through G_4 of uninoculated litters born to C3Hf/Gs female 45818 a sister of the source animals for the experimental series. No leukemias have been observed among 4 litters totalling 25 mice, presently 8 to 10 months of age.

Transmission in other strains of mice. The pattern of congenital transmission of Moloney virus in the *Fg* (high leukemic) and *Lw* (low leukemic) sublines of C3H strain mice is similar to that observed in C3Hf/Gs mice. Transfer of virus through females resulting in lymphocytic neoplasms in the offspring was observed through the G_3 generation, following introduction parenterally at G_0. (Table III).

In contrast, although RFM mice are highly susceptible to this leukemia virus, transmission was observed only through the first generation.

All 4 offspring developed thymic neoplasms and died at 4 months of age, but all had suckled a leukemic mother. Eleven litters (62 mice) of the succeeding G_2 to G_4 generations have remained non-leukemic through 10 months. Attempts were made to detect the presence of virus by foster-nursings of susceptible C3Hf/Gs upon RFM mothers in the G_2 to G_4 generations. These mice also have

TABLE III. Transmission of Leukemia Virus (Moloney) through the Maternal Line of C3Hf/Fg, C3Hf/Lw and RFM Mice.

	No. of Litters	Age of mice at time of infection	No. Mice Leukemic / No. in Litter	Avg Age in Mo at Leukemic Death
C3Hf/FG				
Virus-inoculated litter (G_0)	1	7 days	3[1]/4	6
Subsequent non-inoculated litters				
G_1	1	—	3[1]/5	5
G_2	2	—	3/3, 1/4	5, 6
G_3	3	—	3/9, 2/6, 3/8	4, 4½, 4½
C3Hf/Lw				
Virus-inoculated litter (G_0)	1	7 days	3/3	4
Subsequent non-inoculated litters				
G_1	3	—	1/3, 6/6, 1/2	7, 6, 6
G_2	4	—	2/3, 4/7, 4/7, 4/6	5, 5, 4, 5
G_3	2	—	1/3, 2/4	4½, 5
RFM				
Virus-inoculated litter (G_0)	1	1 day	3/3	2½
Subsequent non-inoculated litters				
G_1	1	—	4[2]/4	4
G_2	1		0[3]/3	—
G_3	5	—	0/3, 0/10, 0[3]/14, 0/11, 0[3]/16	—
G_4	5	—	0/7, 0/5, 0[3]/8, 0/6, 0/6	—

[1] See text for studies of transmission through paternal line, using infected males of these litters.

[2] This litter suckled a leukemic mother.

[3] Foster-nursings of highly susceptible C3Hf/Gs mice upon mothers of these litters did not reveal presence of virus.

remained non-leukemic.

All of the lymphocytic neoplasms appearing in C3Hf/Fg mice, listed in Table III, were thymic in origin; many were thymic lymphosarcomas. The gross and histologic patterns of the virus-induced disease were unlike those observed in the spontaneous disease of this subline in which the thymus apparently is not essential(9). The first spontaneous leukemia among the control mice of this series was observed at 8 months of age.

In several instances C3Hf/Lw and C3Hf/Fg females of the G_1 and G_2 generations (receiving virus from their mothers) were mated with uninoculated males from the breeding colony. The frequency of leukemia among their offspring was similar to that among offspring born to infected mothers mated with infected males.

Studies of Transmission through the Paternal Line. One of the males of a litter of 4 C3Hf/Fg mice (Table 2), inoculated at 7 days with Moloney virus (G_o) developed generalized lymphocytic leukemia with thymic involvement at 4 months of age. Prior to the appearance of the disease, this male was mated with 3 untreated C3Hf/Fg females of the same subfamily and 3 litters (G_1) resulted. Two litters representing G_2 were obtained by mating G_1 litter mates. All of these offspring have remained free from leukemia through 8 months.

Two C3Hf/Fg males of the G_1 generation which received virus as a result of suckling their mother were also mated with untreated C3Hf/Fg females. One of these males became leukemic at 6 months, the other has remained non-leukemic. The resulting 4 litters of the G_2 generation and 2 litters of the G_3 generation are non-leukemic after 8 months.

A litter of 7 C3Hf/Fg mice was born to female 26753. All members of the litter received Moloney virus, preparation LT(V)96 subcutaneously when less than 24 hours old. The 4 females and 3 males developed leukemia, with thymic involvement at 3 to 4 months (average age at death, 3½ months). A litter of 2 males (G_1) was obtained from one of the females and both died leukemic at 3 and 4 months.

These males had suckled a virus-inoculated mother. Three litters of a G_2 generation were obtained, before death of these males, through matings of uninoculated C3Hf/Fg females with the infected males. An additional 7 litters representing the G_3 and G_4 generations, totalling 26 mice, were obtained from matings of litter mates. No leukemias have been observed through the 8 month period preceding spontaneous occurrence of leukemia within this subline.

One of the males of the litter of 7 C3Hf/Fg mice which received Moloney virus parenterally at less than 24 hours of age, was mated to an untreated C3Hf/Fg female and produced a litter of 6 offspring prior to developing leukemia at 4½ months of age. Sib matings within this litter and subsequent litters provided an additional 8 litters (44 mice), constituting the G_1 to G_2 generations. This group has remained non-leukemic through the 8 month observation period.

Reciprocal Foster-Nursing Experiments. To determine the route of transmission of virus from the mother to her offspring, reciprocal foster nursings were carried out. Transmission could occur before birth through extraembryonic membranes to the embryos, after birth through the mother's milk or both before and after birth. It is unlikely that a regular transmission of Moloney virus occurs through gametogenesis and fertilization since the evidence at present indicates that transfer does not occur through the paternal line.

Foster-nursings were accomplished using C3H mothers of 3 different sublines. The litters were allowed to drop through a raised platform constructed of ½″ x ½″ mesh hardware cloth inserted in the bottom of a regular plastic cage. Many of the deliveries were observed and since there was no question of the possibility of litters suckling their own mothers, the adopted procedure was to isolate near-term females at night and collect the young 12 to 15 hours later, or isolate in the morning and collect the litters for foster-nursing at some time during the day. The efficacy of this method of reciprocal foster-nursing has been demonstrated in studies of the transfer of antibodies to polyoma virus(17).

Litters born of virus-inoculated (G_o) or of virus infected mothers (G_1-G_3), that is, infected through the maternal line, were foster nursed on "normal" non-inoculated mothers and *vice versa.* Entire litters were foster nursed.

Two separate experiments rule out the possibility of spread of infection through mouse to mouse contact. Virus-inoculated and non-inoculated litter mates were allowed to remain together from birth through a period which has now reached 9 months. In 3 litters, 6 of 9 virus-inoculated C3Hf/Lw mice became leukemic whereas 11 of the untreated litter mates remain nonleukemic. Four C3Hf/Gs mothers,

TABLE IV. Lymphocytic Neoplasms in Reciprocally Foster-Nursed Mice of 3 C3H Sublines.

Strain	Offspring from "Normal" females Foster-nursed to "Infected" Mothers				Offspring from "Infected" females Foster-nursed to "Normal" Mothers			
	Litter No.	No. of Offspring	No. Leukemic	Avg Age at Leukemic Death Mo	Litter No.	No. of Offspring	No. Leukemic	Avg Age at Leukemic Death Mo
C3H/Lw	1	4	2	3½	—	—	—	—
	2	3[1]	2	5	—	—	—	—
	3	6	6[2]	6	—	—	—	—
	4	4	1	1½	4A	5	0	—
	5	2	1	7	5A	2[4]	1	7
	6	5	4	5½	—	—	—	—
C3Hf/Gs	1	2	1[3]	5	1	5	0	—
	2	6	1	3½	2	4	0	—
	3	6[1]	4	5	3	5	0	—
	4	2[1]	2	5	—	—	—	—
	5	6	0	—	5A	5	1[4]	4
	6	6	4	5	6A	4	0	—
	7	4	3	4½	7A	4	2	5
C3Hf/Fg	1	6	2	4½	1A	4	0	—
	2	8[1]	3	5	—	—	—	—

Survivors among the group of animals in this table are presently 7 to 9 months of age. Litters born to "normal" and "infected" mothers on same morning or evening were interchanged. These are designated in table as 4 and 4A, 5 and 5A, etc.

[1] These litters were foster-nursed upon infected mothers of G_1 or G_2 generation, i.e., those mothers 1 or 2 generations removed from the original virus-inoculated litter. All others were foster-nursed upon mothers which received virus parenterally at birth.

[2] This litter of 6, all of which became leukemic, was foster-nursed upon a virus-inoculated C3Hf/Lw female. The foster-nurse died leukemic at 3½ months of age. Two litters of a succeeding generation were born to 2 of 6 of these foster-nursed mice; 4 of a litter of 7 and 3 of a litter of 7 died leukemic at average ages of 5 and 4 months.

[3] Females of this litter which suckled a virus-inoculated mother, died of leukemia at 5 months of age. A litter of 3 males was born prior to the mother's developing leukemia. Two of 3 of this succeeding litter have died leukemic at 5½ months of age.

[4] These litters were born to leukemic mothers but did not obtain mother's milk before foster-nursing.

which suckled virus-inoculated litters, were allowed to deliver subsequent litters. None of 25 offspring has developed lymphocytic neoplasms. Moloney(19) has obtained evidence in BALB/c mice of the non-contagious nature of this virus.

It is clear from the results in Table IV that this leukemia virus is effectively transmitted through the mother's milk in all 3 C3H sublines studied. Further, it is clear that transmission is as effective by mothers infected originally through suckling as by mothers receiving virus parenterally. Apparently high-leukemic lines may be established by foster nursing of previously unexposed litters upon infected mothers. (See Litter #3, C3Hf/Lw strain and Litter #1, C3Hf/Gs strain).

The results are somewhat equivocal in those offspring born of "infected" mothers and foster-nursed upon "normal" mothers. In 2 instances in which leukemia appeared among the offspring, the mothers exhibited signs of leukemia before births of their litters. More data are desirable to determine if Moloney virus passes the placental or other extra-embryonic barriers. Ida and Moloney(20) have obtained preliminary data indicating such a transfer, however the conditions concerning route and time of inoculation, strain of mouse etc. were quite unlike those reported here.

Discussion. It is clear from the present results employing the highly leukemogenic virus of Moloney that transmission occurs through successive generations of the 3 different C3H strain sublines studied following introduction of the virus in neonatal mice. The most effective route is through the mother's milk. There is every indication that a "low-leukemic" strain may be transformed to a "high-leukemic" strain provided the strain is susceptible to the leukemogenic action of the virus and is capable of transmission of it to the offspring through the maternal line. Infection through 4 successive generations of C3Hf/Gs mice was observed with frequencies and average age at death from the disease being quite similar. RFM mice, although highly susceptible to the leukemogenic action of Moloney virus are apparently not capable of transmitting it to offspring. C58 mice, with a lower susceptibility to Moloney virus, appear also to fall in this category. C57Br/cd mice were found by Gross(10) to be highly susceptible to Passage A strain of his leukemia virus but apparently were unable to transmit it to offspring. A similar failure of inoculated mothers of the AB strain to transfer myeloid leukemia virus to their offspring was reported(21).

Our findings are similar to those of Rubin *et al.*(18) concerning transmission of an avian leukosis virus to the extent that transmission of infection was not achieved through the paternal line.

It is apparent also that a "low-leukemic" line is established simply by foster-nursing mice born to infected mothers upon mothers having no previous contact with virus, providing the foster-nursed litter does not obtain milk from its infected mother. This was achieved in C3Hf/Lw mice.

The results reported here stand in sharp contrast to the facts developed through studies of certain high-leukemic strains of mice such as AKR, C58, F and C3Hf/Fg. All attempts to transform high-leukemic to low-leukemic strains and *vice versa* have proved unsuccessful. Reciprocal foster-nursings(16, 22, 23, 24, 25), successive foster-nursings in AKR mice (24) and transfer of fertilized ova reciprocally between AKR and C3H strain mice(26,27) have been ineffectual. Further, reciprocal matings of high- and low-leukemic strains usually results in populations of F_1 hybrid mice having very similar frequencies and average ages at death from the disease(16,22,23,24,25,28). It is clear therefore that in certain high-leukemic lines the male contributes equally as well as the female to the potentiality of F_1 hybrids to develop leukemia.

The failure of male transmission of Moloney virus and apparently also the Gross virus(10) may be the result of loss of virus from spermatozoa with the shedding of cytoplasm, as suggested for an avian leukosis virus(18). Since precise and rapid *in vitro* and *in vivo* bioassay techcniques are not yet available for these viruses this question cannot presently be resolved.

Only a sporadic occurrence of lymphocytic leukemia was observed in those offspring obtained from virus-inoculated or "infected" mothers and foster-nursed upon mothers having no previous contact with virus. To obtain unequivocal evidence of passage of Moloney virus across extra embryonic membranes, it will be necessary in future experiments to use the strictest of precautions and resort to Caesarean sections. It is clear, however, from our data that Moloney virus is not regularly transmitted via germinal cells. Definitive evidence concerning the Gross virus has not yet been published.

A counterpart may exist to explain the occurrence and transmissibility of a leukemia virus in certain high leukemic strains of mice, particularly AKR, from which the Gross virus is recoverable (from leukemic mice) in an infectious state. The virus which produces CO_2 sensitivity in *Drosophila* may assume alternative states. The stabilized state resembling prophage, can mutate to a form which is unable to produce mature infective virus. The reverse mutation also occurs. This virus in the stabilized non-infectious form is transmitted regularly to succeeding generations through the gametes. A shift to an infectious state however leads to transmissibility by cell-free extracts wherein natural transmission from fly to fly is not observed, and infrequent transmission to progeny occurs(29).

Summary. The pattern of congenital transmission of a leukemia virus was studied in several inbred strains of mice varying in their susceptibilities to virus. Transmission of virus occurred through the maternal line in 3 C3H sublines, 2 low-leukemic and one high-leukemic. RFM mothers failed to transmit infection. There was no indication that parenterally-infected males, of any of the strains studied, transmitted virus to their offspring. Reciprocal foster-nursings of litters born to infected and non-infected mothers revealed that transfer through the mother's milk was the most effective route of transfer. Results of transmission during the prenatal period

were equivocal. Leukemic lines were established in C3Hf/Lw and C3Hf/Fg strains through inoculation of virus into prospective parents in the neonatal period. The frequencies and ages at death from lymphocytic leukemia through 4 generations of C3Hf/Gs mice revealed no loss of virulence of virus. Apparently, low-leukemic and high-leukemic lines may be established simply through foster-nursing of litters. There was no evidence to support the concept for transmission of virus through germinal cells. The results reported here concerning the pattern for congenital transmission of Moloney virus stand in sharp contrast to the patterns for transfer of potentialities to develop leukemia in the high-leukemic strains of mice, AKR, C58, F and C3Hf/Fg.

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Plaque Formation by Myxovirus Parainfluenza Type 2 in Grivet Monkey Kidney Cells. (27046)

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The plaque technique developed by Dulbecco(1) provides a method for relatively precise analysis of infectivity of virus preparations. This technique also lends itself to isolation of pure lines of virus. In many instances plaque morphology has been used to differentiate between virus strains and types(2,3). Deibel has reported plaque formation with parainfluenza type 3 virus in stable human amnion cells(4). Marston and Vaughan have reported a plaque method for parainfluenza type 3 virus assay wherein the plaques are located by the hemadsorption technique(5). Hsiung has reported that the plaque technique may allow detection of some myxoviruses, although cytopathic changes are not apparent in fluid cultures of the virus(6). This report describes adaptation of the plaque technique for parainfluenza type 2 virus in monolayers of grivet monkey kidney cell cultures.

Materials and methods. Viruses. The strain of myxovirus parainfluenza type 2 used throughout these studies was obtained from Dr. Robert M. Chanock and has been propagated in grivet monkey kidney cell cultures in this laboratory. The strain has been freed of contaminating simian viruses. All of the virus samples described here were obtained from infected fluids of grivet monkey kidney cell cultures or purified preparations made from these infected fluids.

Tissue culture. Trypsinized grivet monkey kidney cells were obtained by digestion of the cortical tissue with 0.25% trypsin (Wilson 1:300) overnight at 5°C. The trypsinized cells were harvested by centrifugation and suspended in growth medium (0.5% lactalbumin hydrolysate, 0.025% yeast extract, Hanks' salt solution, and 2% calf serum). The cells were planted in Smith bottles at a concentration of 250,000 cells/ml and 25 ml/bottle. The bottles were incubated at 36-37°C. The medium was removed on the fourth or fifth day and replaced with medium 199 containing 2% calf serum. The cell cultures were ready for plaque tests 7 days after planting.

Agar overlay medium. a) Two per cent agar (Difco, granular). The agar is prepared as required as a 2% (W/V) solution. The agar is sterilized by autoclaving for 30 minutes at 120°C. It is removed from the autoclave, cooled to 45°C, and kept at that temperature until used.

b) A 4.3X concentrate of the nutrient medium is made up as follows.

	ml
Autoclaved demineralized water	300
Eagle's amino acids (100X)*	43
Hanks' salt solution 10X, without glucose, low calcium**	430
Glucose (100 g/l)	43
Glutamine (29 g/l)	43
Sodium bicarbonate (10%)	72.5
Penicillin	430,000 units
Streptomycin	430,000γ
Autoclaved demineralized water	to make 1 liter

c) All components of the medium are sterilized by filtration through Selas 02 or ultrafine glass filters. It is important that this concentrate be prepared by adding each of the ingredients in the order listed. The medium is adjusted to pH 7.5 ± 0.05 with sodium hydroxide.

d) The final overlay medium is prepared by mixing 30 ml of the medium concentrate per every 100 ml of 2% agar at 45°C. Two and twenty-five one hundredth (2.25) ml of neutral red (0.1% W/V) is added for each 130 ml of overlay. It is essential that all materials be thoroughly mixed.

Virus seeding. The medium is removed from each of the cell culture bottles. The cell sheet is washed once with low calcium Hanks' (containing glucose), adjusted to pH 8.0. Cultures are seeded with 0.5 ml of the appropriate virus dilution. Hanks' balanced salt solution at pH 8.0 is used as the virus diluent. The bottles are placed on a level surface in a 36-37°C incubator for 1.5 hours. At the end of incubation period, they are removed from the incubator and 25 ml of final overlay medium pipetted into each bottle in such fashion that the agar does not impinge on the cell sheet directly. The bottles are placed cell sheet down at room temperature until the agar hardens. They are then placed (inverted) in an incubator at 36-37°C. All the plaque cell cultures are covered with aluminum foil to avoid the photodynamic destruction of the cell sheet by neutral red. The plaques are countable and are read in 10-12 days.

Results and discussion. Incubation time. By the method described, excellent plaque formation was obtained with parainfluenza type 2 virus. Typical plaques are shown in Fig. 1. Plaques were not easily discernible 8 days after seeding. By the tenth day of incubation, the plaques were readily visible and could be counted with ease. The number of plaques per bottle usually increased by the twelfth day and the plaques grew somewhat in size. Typical data indicating numbers of plaques per bottle at 10 and 12 days of inclubation are given in Table I.

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* Obtained from Microbiological Associates as a sterile product.

** Anhydrous calcium chloride, 0.7 g/liter.

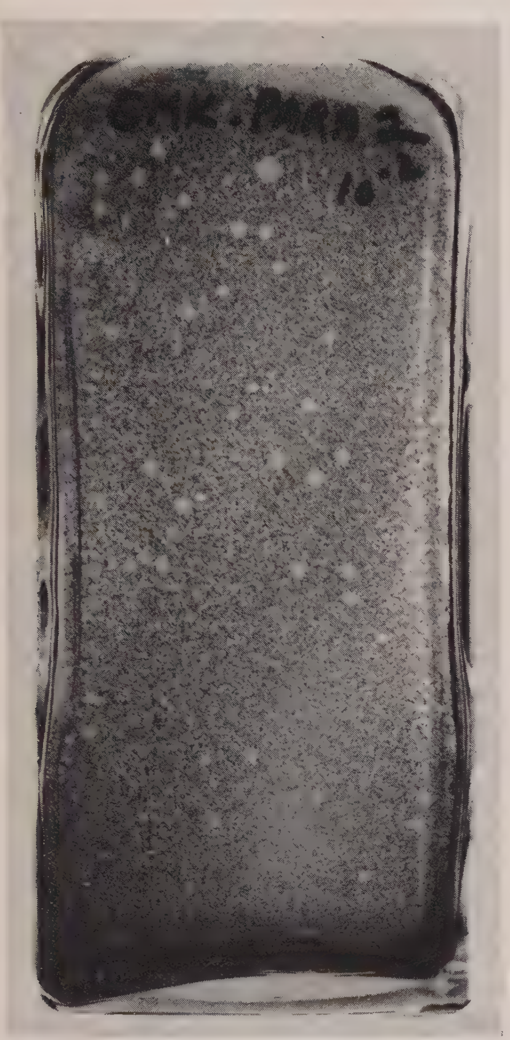

FIG. 1. Plaques of myxovirus parainfluenza type 2 in grivet monkey kidney cell cultures.

TABLE I. Plaque Counts at 10 and 12 days.

Sample No.	10th Day No. of plaques/bottle	12th Day No. of plaques/bottle
1	89, 109	107, 128
2	68, 95	109, 81
3	45, 45	49, 54
4	21, 13	21, 12
5	25, 35	24, 35
6	100, 164	128, 167
7	113, 92	132, 134
8	6, 5	8, 7
9	12, 11	13, 12

Correlation of plaque assay with roller tube hemadsorption assay. The plaque assay, in general, showed good correlation in magnitude with infectivity titers of parainfluenza type 2 virus measured by the hemadsorption endpoint(7). These data are outlined in Table II.

TABLE II. Comparison of Plaque Assay with Roller Tube Hemadsorption Assay.*

Sample No.	PFU/0.2 ml	$TCID_{50}$/0.2 ml
1	1.8×10^6	5.0×10^6
2	$.5 \times 10^5$	1.0×10^5
3	$.6 \times 10^8$	2.5×10^8
4	2.0×10^6	5.0×10^6
5	$.6 \times 10^6$	1.6×10^7
6	$.7 \times 10^5$	1.6×10^6
7	1.1×10^7	1.6×10^7
8	$.6 \times 10^7$	1.6×10^7
9	1.5×10^6	2.5×10^6

* Hemadsorption assays were performed in grivet monkey kidney cell cultures. 0.5 log dilutions were employed. A 0.1% suspension of guinea pig erythrocytes was used. Incubation with red cells for 20 min at 4°C and 2 hr at room temperature.

We are indebted to Dr. Louis Potash for hemadsorption assays.

Proportionality on dilution.† An analysis of the proportionality on 10-fold dilution was made for a series of 58 samples. A 10-fold increment should yield a theoretical ratio of 0.1 or 10%. Geometric mean ratio for the 58 samples analyzed was estimated to be 0.105 or 10.5% with 95% confidence limits of 9.1% and 12.1%. This did not differ significantly from expectation. Individual values ranged from 2.4% to 20.4%.

Summary. A method has been described for plaque formation of parainfluenza type 2 virus in grivet kidney monolayer cell culture. The method has shown utility in the assay of virus infectivity. The plaquing technique should also be useful for cloning of this virus for genetic studies.

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† We are indebted to Mr. Joseph L. Ciminera for statistical analysis of our data.

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A Procedure for Concentration of Normal Urinary Albumin and Globulins.* (27047)

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Normal adult human urine contains an estimated 15-30 mg/day of albumin and an undetermined quantity of protein corresponding electrophoretically to serum globulins(1,2). Webb, Rose and Sehon(3) concentrated urinary albumin by electrophoresis on starch and elution of the appropriate region. Some physical and serological properties were studied and found to correspond to serum albumin. The presence of γ-globulins, predominantly in a fragmented state, has also been unequivocally shown(3,4) in a similar manner. Zone electrophoresis is generally an unsatisfactory procedure for separating γ-globulins in reasonable purity(4,5,6).

This paper describes a procedure for concentration and estimation of albumin and of globulins from normal urine, almost free of extrinsic carbohydrate material. "Globulins" refer here only to those proteins migrating toward the cathode at pH 4.5(7).

Materials and methods. Lyophilized fraction RS-1 of the total nondialyzable urinary solids (8) was the starting material. This is the veronal buffer-soluble, nonultrafiltrable fraction.

A 5% solution of RS-1 was precipitated by saturation with $(NH_4)_2SO_4$. After 2 hr standing in the refrigerator, the mixture was centrifuged (35000 × g × 45 min). Most of the uronic acid in RS-1 was in the clear, colorless supernate, which amounted to 4% of the starting weight of RS-1. The precipitate was redissolved in water, dialyzed free of $(NH_4)_2SO_4$, then relyophilized. If this preliminary precipitation was omitted, the subsequent separation was unsatisfactory.

Five hundred mg of the lyophilized material, corresponding to eight 24-hour urines (from male laboratory workers following their daily routine), was mixed with about 5 ml water and placed on a 21 x 170 mm DEAE-cellulose column (OH form). The column was eluted stepwise; successive eluants were 500 ml portions of water, 0.05 M NaH_2PO_4, and 0.05 M NaH_2PO_4—0.05 M NaCl. Flow rate was 25 ml/hr.

The Ouchterlony agar gel double diffusion technic was used for serological examinations (9).

Results and discussion. The colorless second eluate contained the serum globulins. The third eluate consisted almost entirely of albumin, and on lyophilization was a cream colored, fluffy powder.

Albumin. Yields were reproducible, amounting to 100±10 mg, which represents an excretory rate of about 12 mg/day.

On analysis, a typical sample of the lyophilized material contained 15.4% N, 2.3% hexosamine and 3% hexose, but no hexuronic or sialic acids. Some contamination is also indicated by the electrophoretic pattern (Fig. 1). This contamination was estimated planimetrically at 5—15% in various preparations.

Zone electrophoresis on polyacrylamide gel ("disc electrophoresis" apparatus, Canal Industrial Corp., Bethesda) demonstrated a single sharp band, migrating like serum albumin. This technic has very high sensitivity and resolution, but mucosubstances may not necessarily be stained.

Analytical ultracentrifugation (10 mg in 0.9 ml M NaCl; 56,100 rpm; 134 min) of urinary albumin demonstrated a single gradient, moving in the double sector cell at the same rate as a simultaneously run serum albumin sample.

Serologically, the material gave a single line of identity with serum albumin against rabbit anti-human serum albumin (Sylvana Co., Milburn, N. J.) and also against rabbit anti-human serum (Fig. 2). This is a sensitive indication that the contaminants either are heter-

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† Helen Hay Whitney Foundation Research Fellow.

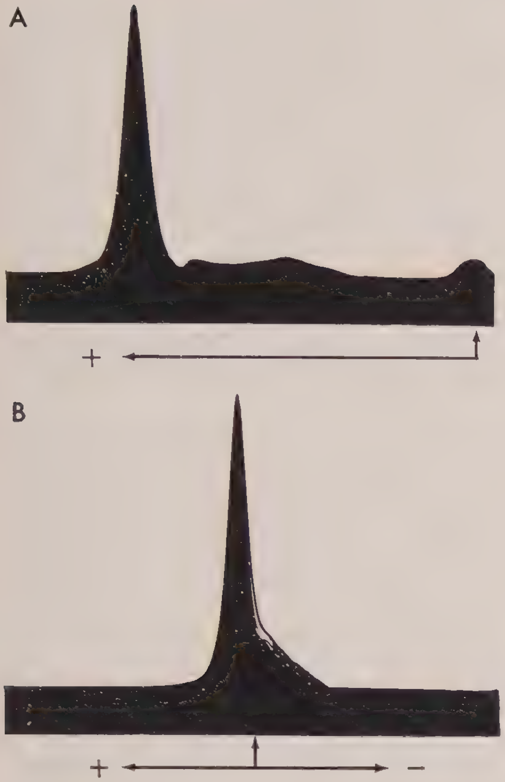

FIG. 1. Electrophoretic patterns of urinary albumin (A) at pH 8.6 (6 mg/ml, veronal, 0.1M, 5.6 v/cm, 1°C, asc. mobility -6.4 cm^2/v sec) and (B) at pH 4.5 (8 mg/ml, acetate, 0.1M, 5 v/cm, 1°C, desc. mobility -0.29 cm^2/v sec) after 4 hr. migration. Spinco model H instrument.

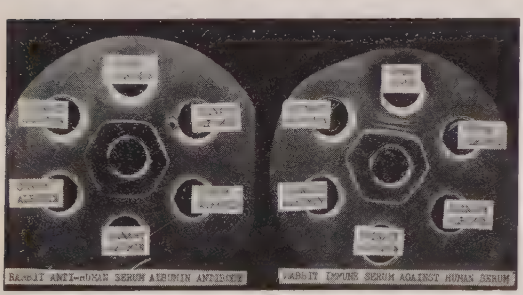
RABBIT ANTI-HUMAN SERUM ALBUMIN ANTIBODY
RABBIT IMMUNE SERUM AGAINST HUMAN SERUM

FIG. 2. Precipitin reaction on Ouchterlony plates. Left: Alternate wells of serum albumin and urinary albumin against rabbit anti-human serum albumin (center well). Right: Wells of human serum (top), serum albumin (top, right) and urinary albumin against rabbit anti-human serum (center well).

ogeneous, nonantigenic, or originate from the genitourinary tract.

The following experiments were intended to determine whether the recovery was quantitative. Albumin equivalent to about 3mg/24 hr urine was present in eluate 2, as estimated from its electrophoretic diagram (pH 8.6). Serologically detectable, but not electrophoretically detectable, quantities of albumin were also present in a fourth eluate, made with 0.05 M NaH_2PO_4—0.1 M NaCl, but not further elutions with 0.05 M NaH_2PO_4—0.25 M NaCl and with 0.1 M NaOH, which removed the rest of the RS-1 from the column (overall recovery by weight, 92% or more).

Two further main nondialyzable fractions remain(8): fraction R-1 (nonultrafiltrable, buffer insoluble), in which albumin could not be detected serologically, and the ultrafiltrate, which has been subdivided into several subfractions(10). A sample of ultrafiltrable subfraction UF-3 was found by immunoelectrophoresis and disc electrophoresis to contain some albumin. After concentration of the albumin by $(NH_4)_2SO_4$ precipitation(10), a quantity estimated to correspond to 0.3 mg/24 hr urine was seen by free electrophoresis, although none was demonstrable by conventional paper electrophoresis (serum albumin control) using Light Green SF dye for visualization; the albumin from RS-1 also stains very poorly with this dye. The other ultrafiltrate subfractions were negative, except for a barely serologically detectable trace in UF-2, presumably adsorbed on this insoluble fraction. Evidently the material isolated from RS-1 represents essentially all the normal urinary albumin.

The estimated average normal excretion of albumin thus totals about 16 mg/24 hr urine, three-fourths of which is isolated by the foregoing procedure.

Globulins. The lyophilized second eluate was suspended in 5 ml water, and dialyzed against a half-saturated $(NH_4)_2SO_4$ solution for 48 hours in the cold. After removal of the precipitate, the supernate was dialyzed against saturated $(NH_4)_2SO_4$. The resulting precipitate was removed and the supernate and 2 precipitates dialyzed, lyophilized, weighed, analyzed (Table I) and examined electrophoretically(2). As expected(11), all the globulins were in the first precipitate. The second precipitate contained some albumin, as mentioned above, and also material migrating in the α_2 to γ region at pH 8.6, but at pH 4.5 only material

TABLE I. Analyses of Eluate 2, in Per Cent of Lyophilized Dry Weight.

	Total eluate 2	Ppt. sat/2-$(NH_4)_2$-SO_4	Ppt. sat-$(NH_4)_2$ SO_4	Supernate
Mg recovered	197	57	99	26
Hexose[*]	13.3	8.2	14.9	24.1
N	14.41	15.92	13.35	8.04
Hexosamine[†]	7.4	2.7	5.1	6.7

[*] Anthrone method; standard was equal weights galactose and mannose.

[†] As glucosamine base.

migrating like albumin was seen. The supernate contained material which moved in the α_2 to γ region at pH 8.6, being a broad peak centered in the β region. Evidently, on separation as described, some further portion of RS-1 had become soluble in saturated $(NH_4)_2SO_4$.

The analyses (Table I) as compared with serum globulins(12,13), indicate some contamination with mucosubstances of the "serum globulin" fraction precipitated by half saturation with $(NH_4)_2SO_4$. However, all detectable material behaving like serum globulins at pH 4.5 was present in this fraction, and in 70-fold concentration as compared with the total nondialyzable solids(8). Five components, including some albumin, were seen by disc electrophoresis. Incomplete solubility of the fraction in water and solubility in dilute salt solutions confirms the presence of globulins. The results indicate that normal excretion of recognized serum globulins (including γ globulins) does not exceed about 6 mg/day.

Summary. A procedure for concentration of albumin and globulins from normal urine is described. The albumin appears to be identical with serum albumin, and excretory rate is about 16 mg/day, under conditions of normal activity. Excretion of globulins amounts to less than 6 mg/day.

We thank Dr. Karl Schmid, Harvard Med. School, for the ultracentrifugation and Dr. G. Grant, Shrewsbury, England, for immunoelectrophoretic examination of fraction UF-3.

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Alternate Pathways of Glucose Metabolism in *Azotobacter agilis*.[*] (27048)

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Johnson, Sobek, and Clifton(1) found that more C_2-carbon from glucose-2-C^{14} than C_1-carbon from glucose-1-C^{14} was assimilated by *Azotobacter agilis*, suggesting that a pentose pathway, involving an immediate decarboxylation, might be of quantitative importance in the metabolism of this organism. The presence of an active glucose-6-phosphate dehydrogenase had been demonstrated by Mortenson and Wilson(2,3). Extracts used by these workers also oxidized 6-phosphogluconate (6-PG) rapidly. However, they were unable to demonstrate reduction of triphosphopyridine nucleotide (TPN) or diphosphopyridine nucleotide (DPN) in presence of 6-PG and found no stimulation by TPN of oxygen uptake on 6-PG. Mortenson, Hamilton, and Wilson(4) found that 6-PG was split quantitatively to pyruvate and glyceraldehyde-3-phosphate (G-3-P), presumably following a dehydration reaction. The work to be presented here represents a reinvestigation of the possibility of 6-PG dehydrogenase activity in *A. agilis* and a study of other reactions connected with 6-PG dissimilation.

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[*] This investigation was supported in part by a grant from Nat. Inst. of Allergy and Infectious Diseases, P.H.S.

Materials and methods. For spectrophotometric experiments, cells of *A. agilis* were grown on a shaker in 2-liter flasks containing 1 liter of Burk's nitrogen-free medium with 2% glucose or sucrose. Sucrose-grown cells were harvested at 24 hours. A shorter growth period of 16 hours was used with glucose-grown cells to avoid gum formation. The cells were collected by centrifugation, washed twice with distilled water, suspended in 0.05 M tris (hydroxymethyl)-aminomethane (Tris) buffer at pH 8.0 at a concentration of about 2 g in 8 ml, and then disrupted by sonication for 15 minutes in a 9-kc Raytheon Sonic Oscillator. Whole cells and debris were removed by 2 centrifugations at 2,000 g for 10 minutes. The supernatant fluid from the final centrifugation is designated as the crude extract. Fractionation of the crude extract was accomplished by centrifugation for 120 minutes at 144,000 g in the Spinco Model L Ultracentrifuge. The supernatant fluid from this centrifugation, designated the 144s120 fraction, was the source of enzyme for spectrophotometric assays, unless otherwise indicated.

Assays were performed with the Beckman Model DU Spectrophotometer by measurement of increase in optical density at 340 mμ. Unless otherwise indicated, cuvettes contained 1.5 ml glycyl-glycine buffer (0.4 M, pH 7.4), 0.2 ml TPN or DPN (2 mg/ml), 0.1 ml of 0.1 M $MgCl_2$, 0.1 ml enzyme preparation (about 200 μg protein), and 0.6 ml distilled water. TPN (98%-100% pure) and DPN (95%-100% pure) were obtained from Sigma Chemical Co. After taking a zero reading, 0.1 ml of 0.02 M substrate was added, and readings taken at 15- or 30-second intervals. No change in optical density occurred in absence of substrate.

For manometric experiments, cells were grown as previously described, and the crude extract was prepared in the same manner except that concentration of cells was reduced to 1 g of cells per 10 ml of suspending medium. Fractionation procedures included recovery of the particulate fraction (144p120) after ultracentrifugation, and homogenation of the particle in 0.05 M Tris, pH 7.2. The volume of Tris used for homogenation was calculated so that 0.2 ml of suspension would be equivalent to 1 ml of crude extract.

Oxygen uptake was measured in the Warburg constant volume respirometer at 30°C.

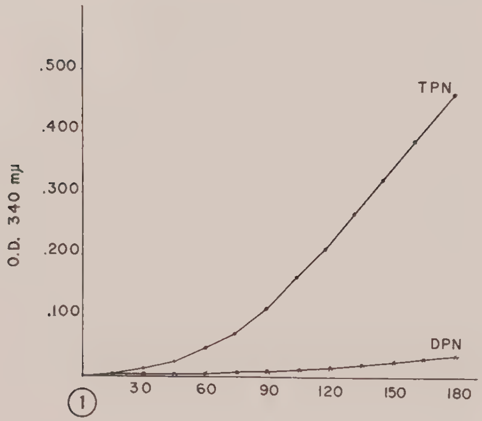
Results. Crude extracts of *A. agilis* showed no reduction of TPN in presence of 6-PG, even when cyanide was added. The 144s120 fraction of the crude extract, however, catalyzed a rapid reduction of TPN (Fig. 1). Rate of reduction of DPN was much slower than that observed with TPN.

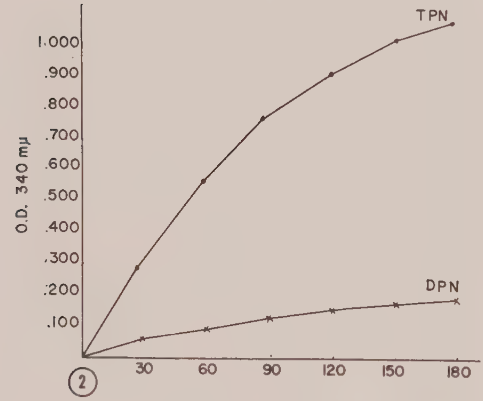
TABLE I. Effect of TPN on Oxidation of 6-Phosphogluconate by Crude Extracts of *Azotobacter agilis*.*

System	Total Oxygen Uptake	
	15 min	30 min
	μl	μl
Endogenous	4	12
" with TPN	1	10
6-Phosphogluconate	22	53
" with TPN	45	64

* Flask contents: Main compartment—1.0 ml crude extract, 0.3 ml phosphate buffer (0.66 M, pH 7.0), 0.1 ml $MgSO_4$ (0.1 M), Tris buffer (0.05 M, pH 7.2) to a total vol of 2.2 ml; Sidearm—where indicated 0.2 ml TPN (2 mg/ml), 0.3 ml 6-phosphogluconate (0.02 M); Center well—0.1 ml 10% KOH.

Table I shows the stimulation of TPN of the oxidation of 6-PG by a crude extract. This effect could be demonstrated only under carefully controlled conditions. Inconsistent results were obtained when the cells used were more than 24 hours old or when the cell suspensions used for preparation of extract contained much more than 1 g (wet weight) of cells per 10 ml of suspending medium. It was necessary also to take readings of oxygen uptake at intervals shortly after mixing of extract and substrate, as differences between flasks with and without TPN were obscured in about 30 minutes. TPN was found to stimulate crude extracts of both glucose- and sucrose-grown cells.





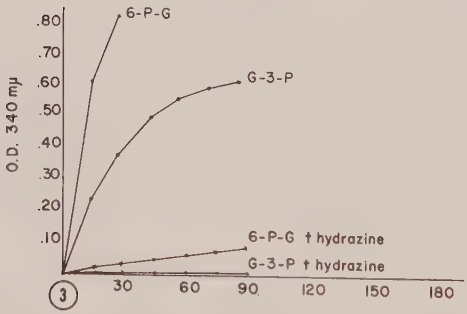


FIG. 1. Reduction of TPN and DPN in presence of 6-phosphogluconate by 144$_{s}$120 fraction from *Azotobacter agilis.*

FIG. 2. Reduction of TPN and DPN in presence of glyceraldehyde-3-phosphate by the 144$_{s}$120 fraction from *Azotobacter agilis.*

FIG. 3. The effect of hydrazine on TPN reduction in presence of gyceraldehyde-3-phosphate and 6-phosphogluconate. Substrate, hydrazine, and enzyme were combined first, and reaction started by addition of TPN.

No reduction of TPN or DPN by the 144s120 fraction occurred with pyruvate as substrate. The results with G-3-P are shown in Fig. 2. With G-3-P a rapid reduction occurred with TPN, but DPN served only poorly as hydrogen acceptor. Good activity with G-3-P occurred when extracts were prepared from cells that had been frozen for several months. Once prepared, however, the extract lost its activity rather rapidly and no TPN reduction could be demonstrated with extracts more than 2 days old.

Attempts to fractionate the 144s120 preparation further with ammonium sulfate to separate the 6-PG and G-3-P activities were unsuccessful. Almost all of the activity on 6-PG was lost on fractionation.

Fig. 3 shows the effect of 0.12 M hydrazine on TPN reduction when 6-PG and G-3-P served as substrate. The reduction of TPN was completely inhibited with G-3-P, whereas an extensive, but not complete, inhibition was noted with 6-PG.

The sodium salt of *p*-chloromercuribenzoate was also tested. At a concentration of 1×10^{-3} M complete inhibition of activity on both G-3-P and 6-PG was observed.

The 144s120 fraction was tested for aldolase by addition of fructose-1,6-diphosphate. Aldolase would yield G-3-P which the extract would oxidize with the reduction of TPN. However, no TPN reduction occurred. To test the possibility that reoxidation of TPN was obscuring the result, crystalline aldolase and fructose-1,6-diphosphate were added to the system. TPN reduction then occurred at the same rate as with G-3-P, showing that no such reoxidation was occurring. The particulate fraction was tested for aldolase activity by incubation with fructose-1,6-diphosphate, heating to destroy enzymes which reoxidize reduced TPN, and then measurement of TPN reduction upon the addition of 144s120 fraction. No TPN reduction occurred.

Triose phosphate dehydrogenase activity at various hydrogen ion concentrations showed an optimum at pH 8.5, with 80% of maximum activity at pH 7.5 and 50% at pH 9.5. Activity was not increased by pre-incubation with cysteine.

In an investigation of the mode of oxidation of reduced TPN, incubation of reduced TPN with crude extract resulted in a very low rate of reoxidation (Table II). Addition of DPN greatly increased this rate, demonstrating the

TABLE II. Oxidation of Reduced Pyridine Nucleotides by Crude Extract of *Azotobacter agilis.*

Substrate	Decrease in OD at 340 mμ between 60 and 90 sec
TPNH	.030
TPNH + DPN	.135
DPNH	.200

Each cuvette contained: 1.0 ml Tris buffer (0.05 M, pH 7.2), 0.1 ml $MgCl_2$ (0.1 M), 0.3 mg DPN (where indicated), 0.3 mg TPNH or DPNH, sufficient distilled water to bring final volume to 3.0 ml. The reaction was started by addition of 0.2 ml crude extract.

presence of TPN-DPN transhydrogenase. This table also shows the presence of DPNH oxidase in the extract.

Discussion. Our results agree with those of Mortenson and Wilson(3) with regard to the lack of demonstrable TPN reduction with 6-PG as substrate, even in the presence of cyanide, when crude extract was used as a source of enzyme. The fact that we were able to show a rapid TPN reduction, using the fractionated extract, indicates that the crude extract catalyzed a rapid reoxidation of the reduced TPN. The shape of the curve in Fig. 1, representing an increase in the rate with time, suggested the possibility of some other reaction(s) preceding the dehydrogenation. Since Mortenson, Hamilton, and Wilson(4) reported that their extracts split 6-PG to pyruvate and G-3-P, we tested the possibility that one of the latter products was actually the compound which was being dehydrogenated. The results shown in Fig. 2, the fact that TPN reduction proceeded more rapidly with G-3-P than with 6-PG, and the additional finding (Fig. 3) that pre-incubation of enzyme and substrate eliminated the lag we had observed in rate of TPN reduction with 6-PG, suggest that the reduction we were observing with 6-PG actually resulted from dehydrogenation of G-3-P formed from 6-PG by the splitting system. The fact that activity on both G-3-P and 6-PG is inhibited by *p*-chloromercuribenzoate also indicates that there may be a common enzymatic reaction involved. The extensive inhibition by hydrazine of TPN reduction with 6-PG strengthens this interpretation, since hydrazine completely inhibited TPN reduction with G-3-P. The low level of activity observed with 6-PG in the presence of hydrazine may, however, reflect another route of dissimilation of this substrate. Pyruvate is apparently not involved, since no TPN reduction occurs with this substrate.

The finding of a TPN-dependent triose phosphate dehydrogenase in this organism led us to a reexamination of the possibility of the operation of the Embden-Meyerhof pathway. Mortenson and Wilson(2) suggested that this pathway might be quantitatively unimportant because their extracts showed no phosphohexokinase activity. They did find indirect evidence for aldolase. We were unable to show any aldolase activity in our preparations.

The Azotobacter triose phosphate dehydrogenase is similar to that isolated from green leaves(5) in its pH optimum. It differs from the plant enzyme, however, in that activity is not increased by pre-incubation with cysteine. A similar enzyme from *Alcaligenes faecalis* has been described by Brenneman and Volk (6). The purified enzyme in this case required arsenate or phosphate.

In view of the major role played by TPN in the metabolism of *Azotobacter agilis,* we investigated the possibility of a mode of oxidation of reduced TPN other than the TPN-DPN transhydrogenase system. We found no evidence for a direct TPNH-oxidase. However, transhydrogenase and DPNH-oxidase were present in the crude extract.

Summary. Fractions from *Azotobacter agilis* reduce TPN in the presence of 6-phosphogluconate. This reduction is due largely although perhaps not entirely, to oxidation of glyceraldehyde-3-phosphate formed by the splitting system described by Mortenson, Hamilton, and Wilson. This organism possesses a TPN-linked triose-phosphate dehydrogenase, similar to that found in green leaves. The reduced TPN is reoxidized via the TPN-DPN transhydrogenase system.

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Aerogenic Immunization of the Monkey and Guinea Pig with Live Tularemia Vaccine. (27049)

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Active immunization against airborne infection by inhalation of attenuated microorganisms has been proved with experimental animals(1-7). The potential for mass immunization of man by aerogenic vaccination with single or combined live vaccines has been recognized in the USSR(4, 5). In the U.S.A., aerogenic vaccination has not received widespread attention. The present study was initiated to explain the role of 2 routes of vaccination, dermal and respiratory, on the clinical acceptability and immunogenicity of live tularemia vaccine(8, 9) for the monkey and guinea pig.

Materials and methods. The monkeys *(Macaca mulatta)*, weighing between 1.8 and 3.7 kg, were distributed so that each group included animals of comparable weight and sex. The male to female ratio was approximately 3:1. Hartley strain guinea pigs were selected without regard to sex and weighed 475-525 g each. *Pasteurella tularensis* live vaccine strain LVS was cultivated in modified casein partial hydrolyzate (MCPH) liquid medium(8) and suspended in sterile half-strength medium. Aerogenic vaccination with strain LVS and subsequent respiratory challenge with highly virulent *P. tularensis* strain SCHU S5(10) via the respiratory route were performed as described previously(9). For dermal vaccination, 0.2 ml of either a gel-saline (0.1% gelatin and 0.85% sodium chloride) dilution of an MCPH culture of strain LVS or of rehydrated lyophilized LVS live tularemia vaccine, was administered subcutaneously (sc) on the right rear leg of the guinea pig and intracutaneously on the back of the monkey. Monkeys received a respiratory challenge of 750 cells 50 days after vaccination. Guinea pigs received a respiratory challenge of 4×10^3 cells or a sc challenge of 10^3 cells of strain SCHU S5 in the left rear leg 30 days after vaccination. Rectal temperatures of 10 guinea pigs in each group of 40, and of all monkeys, were recorded once or twice daily for 3 days before and 11-15 days after vaccination. Temperatures of all monkeys were recorded for 15-30 days after challenge. Clinical examination of monkeys included a study of chest roentgenograms taken daily for 10 days after vaccination and then every other day until 22 days after challenge. Agglutinin(11) and precipitin(12) titrations were performed on sera obtained 24 days after vaccination from the 10 guinea pigs per group used for the temperature study and from all monkeys 1, 3, and 6 weeks after vaccination. One ml of blood from each monkey was cultured for *P. tularensis* daily for 7 days after challenge and every other day thereafter if the animal remained febrile. Dead animals were autopsied, examined for gross pathology, and the heart blood, lungs, and spleen cultured for *P. tularensis*.

Results and discussion. Live vaccine in the monkey. The simian experiments and their major results are outlined in Table I. Live

TABLE I. Effect of Route of Vaccination on Resistance of the Monkey to Respiratory Challenge with *Pasteurella tularensis.**

Vaccination	No. of animals	Animals yielding blood culture(s) of challenge strain, %	Survival, %
Intracutaneous, 10^8 viable cells rehydrated lyophilized vaccine	8	50	63
Intracutaneous, 10^6 viable cells culture vaccine	7	71	57
Aerogenic, 10^6 viable cells culture vaccine	8	13	88†
None	8	88	13

* All animals challenged with 750 cells of strain SCHU S5.

† Cause of death in the only animal that died in this group was complicated by peritonitis not attributed to tularemia.

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vaccine was administered intracutaneously to 2 groups of animals. A third group received live vaccine aerogenically. With few exceptions, animals vaccinated intracutaneously became febrile (104-105°F) for 12-48 hr 3-4 days after vaccination. In contrast none of the monkeys vaccinated via the respiratory route became febrile. Rehydrated lyophilized vaccine and vaccine culture administered intracutaneously could not be differentiated on basis of skin reactivity. The vaccine lesions were benign but of greater intensity than those resulting from live vaccine administered by acupuncture(13). Chest roentgenograms showed no changes caused by vaccination. All vaccinated monkeys developed *P. tularensis* agglutinins. In general, aerogenic vaccination resulted in higher agglutinin titers (1:320-1:1,280) than did intracutaneous vaccination (1:20-1:320). Aerogenic vaccination resulted in more and denser precipitin lines on agar double diffusion plates than did sc vaccination(12).

Respiratory challenge of vaccinated and nonvaccinated monkeys was performed 7 weeks after vaccination. All animals exhibited evidence of infection. With the exception of 1 control, all animals became febrile within 7 days after challenge. The exception did not exhibit overt disease but developed a diagnostic agglutinin titer. The incubation period did not vary appreciably in the 4 groups of animals, nor did the time of death. Positive blood cultures were obtained from fewer of the animals vaccinated by the respiratory route than from other animals. Roentgenograms indicated that pathologic pulmonary changes were less severe in this group. Also, these animals exhibited fewer and less extensive signs of physical disability than animals vaccinated by the dermal route.

Survival in animals vaccinated aerogenically was 88% in comparison with 57-63% in animals vaccinated intracutaneously and 13% in nonvaccinated controls. Only 1 of 8 animals vaccinated by the respiratory route died subsequent to challenge. The cause of death in this animal was complicated by a perforated colon, not attributable to tularemia, which resulted in peritonitis. These results are in agreement with a previous study in which 9 of 17 monkeys vaccinated by the intracutaneous route survived a virulent respiratory challenge of 1,000 or more organisms and all nonvaccinated animals died(13).

Data on survival of vaccinated monkeys after respiratory challenge together with clinical observations obtained during the course of disease show that aerogenic vaccination with live vaccine afforded comparable if not greater protection against pulmonary tularemia than vaccination by the intracutaneous route.

Live vaccine in the guinea pig. Groups of 40 guinea pigs each were vaccinated by the respiratory or sc route with 10-20, 10^3, or 10^5 cells of live vaccine culture. Sixty % of the animals inhaling 10-20 cells of live vaccine and 100% of those receiving 10^3 or 10^5 cells became febrile 6-10 days later. After sc vaccination none of the animals inoculated with 10-20 cells became febrile whereas 60% of those administered 10^3 cells and 100% of those having received 10^5 cells exhibited fever in 4-8 days. Vaccine by either route proved innocuous except for a febrile response of 1-5 days duration and a benign skin lesion in animals inoculated sc. Table II shows that a greater number of animals vaccinated by the respiratory route developed *P. tularensis* agglutinins than animals vaccinated sc; also, the quantitative serological response of these animals was higher than observed in animals vaccinated sc.

TABLE II. Agglutinin Response of the Guinea Pig 24 Days after Administration of Live Tularemia Vaccine.

Viable cells in vaccine inoculum	Route of vaccination			
	Respiratory		Subcutaneous	
	No. animals	Titer	No. animals	Titer
10-20	4	Neg	9	Neg
	1	1:80	1	1:160
	4	1:160	—	—
	1	1:320	—	—
10^3	1	Neg	4	Neg
	2	1:80	2	1:80
	2	1:160	3	1:160
	1	1:320	1	1:320
	3	1:640	—	—
	1	1:1,280	—	—
10^5	6	1:320	1	1:10
	2	1:640	1	1:20
	2	1:1,280	1	1:80
	—	—	6	1:160
	—	—	1	1:320

Respiratory challenge of one-half of each group of 38-40 guinea pigs was performed 30

TABLE III. Effect of Route of Vaccination on Resistance of Guinea Pig to Respiratory or Subcutaneous Challenge with *Pasteurella tularensis*.

Viable cells in vaccine inoculum	Respiratory challenge Survival on indicated day after challenge, %*				Subcutaneous challenge Survival on indicated day after challenge, %*			
	Respiratory vaccination		Subcutaneous vaccination		Respiratory vaccination		Subcutaneous vaccination	
	7	15	7	15	7	15	7	15
10^5	100	60	30	5	100	90	90	30
10^3	75	45	35	0	100†	68	89†	11
10-20	30	0	5	0	75	15	45	10

* 1-4 x 10^3 cells of SCHU S5; no survival in non-vaccinated animals 7 days after challenge.
† 19 animals per group, all others 20 per group.

days after vaccination with approximately 4 x 10^3 cells of strain SCHU S5. The remaining animals received 10^3 cells of the same culture sc. Two additional groups of 20 nonvaccinated animals each were challenged either by the respiratory or sc route and served as controls.

Table III shows that aerogenic vaccination provided greater protection against both respiratory and sc challenge than did vaccine administered sc. All nonvaccinated animals were dead within 1 week after challenge. Aerogenic vaccination with 10^5 cells protected 60% and 90% of the guinea pigs against the respiratory and sc challenge, respectively, whereas sc vaccination with the same dosage resulted in only 5% and 30% survival, respectively, 15 days after challenge. Lower order survival comparable in pattern was observed in animals that received a vaccine dosage of 10^3 cells. Vaccination with as few as 10-20 viable cells resulted in a low grade immunity demonstrated by survival of a small percentage of the animals or by an increase in survival time.

These data support results obtained with the monkey. The combined animal data strongly suggest that aerogenic vaccination affords greater immunity against tularemia than does vaccination by the dermal route.

Summary. Studies were made on the clinical acceptability and immunogenicity of live tularemia vaccine administered to the monkey and guinea pig by the dermal or respiratory route. Aerogenic as well as dermal vaccination proved innocuous. Animals vaccinated aerogenically showed higher levels of antibody and comparable if not greater protection against challenge with a highly virulent strain of *P. tularensis* than animals vaccinated dermally.

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Increased Lung Serotonin and Pulmonary Edema in Mice Injected with Antiserum to Ehrlich's Ascites Tumor.* (27050)

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Redfern(1) described reactions of guinea pigs injected with rabbit sera containing Forssman antibodies. The guinea pigs showed symptoms resembling those of anaphylaxis but the condition of their lungs was markedly different from the lungs of anaphylactic animals. The lungs of the guinea pigs killed by the heterophile antisera were hemorrhagic and edematous and fluid often extended into the trachea and exuded from the nose. We have recently observed a similar condition in mice injected with serum from a rabbit immunized with Ehrlich's ascites tumor cells (EATC) grown in tissue culture(2). An additional finding in our experiments, however, is the presence of a markedly elevated serotonin content in the lungs of the test animals.

Materials and methods. A rabbit received an intravenous injection of 1 x 10^4 EATC administered every fourth day for 12 days. Thereafter, 1 x 10^7 EATC mixed with Freund's adjuvant were injected subcutaneously twice a week. Eight weeks after initial injection, the rabbit was bled and its serum found to be toxic for mice (see below). Additional doses of EATC were administered weekly or bi-weekly, and the serum used in the experiments to be described was obtained 5 months after initial injection of EATC. This serum had a cytotoxic titer of 1:50 when tested against EATC *in vitro*.

Swiss-Webster, 20-25 g female mice were injected intraperitoneally with 5 billion *B. pertussis* organisms since this procedure increases the susceptibility of these animals to the lethal effects of serotonin, histamine, and anaphylaxis(3). Five to 8 days after inoculation with *B. pertussis,* mice were injected intravenously with 0.5 ml of the rabbit antiserum to EATC. Other pertussis inoculated mice were challenged with 0.5 ml of normal rabbit serum or 0.5 ml of rabbit antiserum to mouse globulin. The latter antiserum had an LD_{50} similar to that of the antiserum to EATC, and killed mice (reversed passive anaphylaxis) in a similar time period (5-15 minutes). Lungs were removed upon death of the animals, weighed, and analyzed for histamine by the method of Shore *et al.*(4), and for serotonin by the method of Bogdanski *et al.*(5).

Results. A few minutes after administration of rabbit antiserum to EATC the mice became still, then prostrate, went into convulsions, and died gasping for air. A large percentage of the animals had bloody, frothy fluid exuding from the nose, and had lungs that were larger than normal with patchy hemorrhagic areas on their surface interspersed with areas of emphysema. Histologic studies confirmed these gross observations.

Hemorrhagic edematous lungs are not seen in mouse anaphylaxis. Indeed, Treadwell *et al.* (6), in describing the widespread visceral congestion in anaphylactic mice, made a point of mentioning the paucity of lung changes in this species. The data in Table I indicate that the mean lung weight of mice injected with antiserum to EATC was significantly greater (350 ± 15 mg) than the mean lung weight of anaphylactic mice (212 ± 17 mg) or mice injected with normal rabbit serum (182 ± 7 mg).

The data in Table I also demonstrate a 10-fold increase in serotonin in the lungs of mice injected with the antiserum to EATC as compared with mice injected with normal rabbit serum. Total lung serotonin of the mice injected with rabbit antiserum to EATC was also significantly greater (4.09 ± 0.51 μg) than the lung serotonin of mice injected with rabbit antiserum to mouse globulin (1.00 ± 0.09 μg). In contrast, there were no significant differences in the total lung histamine content of the various groups of mice.

Discussion. On the basis of the data presented there seems little doubt that hemorrhagic edematous lungs with a high serotonin

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content are not characteristic of reversed passive anaphylaxis in the mouse. As already mentioned, Redfern(1) differentiated guinea pig anaphylaxis from the reaction of these animals to Forssman antibody (hemorrhage and edema of the lungs). Recently, Humphrey(7) observed that Forssman antibodies would not cause mast cell degranulation in the guinea pig although this occurs in anaphylaxis. Also, blood taken just before death of animals injected with Forssman antibodies contained no detectable histamine. Humphrey(7) quotes unpublished experiments of Mongar which indicate that no histamine is released when Forssman antibodies are perfused through the isolated guinea pig lungs. Forssman antigen is present in many tissues of the mouse including the lung(8) as well as in certain mouse tumors(9), but it is doubtful that Forssman antibodies are responsible for the mouse lung pathology which we have observed. Our doubt is based on the fact that to this date, we have been unable to absorb out the lethal effects of rabbit antiserum to EATC with sheep red blood cells. This serum has likewise proved to be nonlethal for guinea pigs. Absorption of rabbit antisera to EATC with various mouse tissues is now in progress. Thus far, the only other rabbit antiserum which has produced hemorrhagic edematous lungs in mice is that of a rabbit injected with mouse mast cell tumor P 815†. A rabbit antiserum to normal mouse spleen cells killed mice but did not produce hemorrhagic edematous lungs. Possible differences in the ability of malignant mouse cells and normal mouse cells to produce rabbit antisera with the property of damaging mouse lungs are now under investigation. The association of pulmonary edema with increased lung serotonin has also been noted by Skillen *et al.*(10) in rats exposed to ozone.

Although the role of serotonin in the etiology of pulmonary edema is still in doubt, preliminary experiments in our laboratory have shown that cyproheptadine, a compound with antiserotonin activity(11), can block the pulmonary edema and hemorrhage produced by rabbit antiserum to EATC.

Summary. Mice injected with a rabbit anti-

TABLE I. Serotonin and Histamine Content of Lungs of Pertussis Inoculated Mice Injected with Various Rabbit Antisera.

Serum Injected	No. of animals	Lung wt. (mg)	Serotonin Content µg/lungs	Serotonin Content µg/g lung	Serotonin Content µg/100g body wt.	No. of animals	Lung wt. (mg)	Histamine Content µg/lungs	Histamine Content µg/g lung	Histamine Content µg/100g body wt.
None (Control)	5	186 ± 9.0	.48 ± .08	2.59 ± .37	2.40 ± .40	5	203 ± 7.5	.13 ± .01	.64 ± .04	.54 ± .06
Normal rabbit serum	4	182 ± 7.5	.54 ± .09	2.91 ± .53	2.57 ± .46	5	196 ± 7.5	.15 ± .02	.76 ± .08	.65 ± .06
Antiserum to mouse globulin	5	212 ± 17.2	1.00 ± .09*	4.80 ± .54*	5.50 ± .39*	5	209 ± 13.4	.17 ± .02	.81 ± .09	.72 ± .08
Antiserum to EATC	5	350 ± 14.7*‡	4.09 ± .51*‡	11.78 ± 1.65*‡	20.44 ± 2.89*‡	5	335 ± 31.5*‡	.13 ± .01	.42 ± .07‡	.57 ± .07

Each number is mean ± standard deviation of mean. * $P < .01$—compared to control. ‡ $P < .01$—compared to antiserum to mouse globulin.

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† Obtained through the courtesy of Dr. Michael Potter, Nat. Cancer Inst., Bethesda, Md.

serum to Ehrlich's ascites tumor developed hemorrhagic edematous lungs with a markedly increased serotonin content. Such lung findings were not seen in mouse anaphylaxis.

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Excretion into Bile of Intrinsic Factor and Its Vit. B_{12} Complex Following Intravenous Injection. (27051)

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It has been shown *in vitro*(1,2,3) that uptake of cyanocobalamin (B_{12}) by liver tissue is enhanced by intrinsic factor concentrate (IF), but whether this phenomenon has any bearing on the biological function of naturally occurring IF is not known. We demonstrated that $Co^{60}B_{12}$, when premixed with IF and injected to rats intravenously, was taken up by the liver in almost absolute preference to other organs(4). It was also shown that degree of the preferential uptake might be related to purity of IF but not to the abundance of reticuloendothelial cells, and that formation of an IF-B_{12} complex was prerequisite to this phenomenon. Our observation has been confirmed by others(5,6). Since IF used in these studies was of hog origin and therefore a heterologous protein to the animals, interpretation of the results seems to be limited because of the possibility of nonspecific uptake of such proteins by the liver. Our further attempt to clarify the mechanism by which the liver captures IF-B_{12} complex has disclosed that part of the complex is immediately excreted into bile following the trapping by the liver, and that the same is true of IF without B_{12}. These findings will not only throw light on the mode of action of IF in hepatic uptake of B_{12}, but will provide a clue as to the mechanism of disposal of foreign substances by the liver. Such data are reported here.

Materials and methods. Young adult rats of Wistar strain which had been raised on a stock diet and rabbits of about 2 kg that had been fed a soy-bean curd diet were used. The IF used was a hog stomach preparation (WES #727)*, and the activity was such that 1 to 2 mg was active in pernicious anemia patients when administred together with 15 μg of B_{12}. The B_{12} binding capacity of this preparation was 500 mμg/mg as measured by dialysis method. The $Co^{60}B_{12}$ used had a specific activity of approximately 0.8 μcurie per μg. Rats were anesthetized with ether and intraperitoneal injection of urethan, 1 ml per 100 g body weight; no anesthesia was given to rabbits. Laparotomy was performed and a glass cannula was inserted into the common bile duct and was tied with the duct. Bile was collected directly from the cannula in the case of rats, or through a polyethylene tubing in the case of rabbits. After securing the basal bile for half an hour, a saline solution of IF or saline mixture of IF and $Co^{60}B_{12}$ was injected intravenously, the tail vein being used in the former and the ear vein in the latter. The amounts of IF and $Co^{60}B_{12}$ to be mixed were so adjusted that the binding power of IF was always in excess of $Co^{60}B_{12}$ in order to have $Co^{60}B_{12}$ all in bound state.

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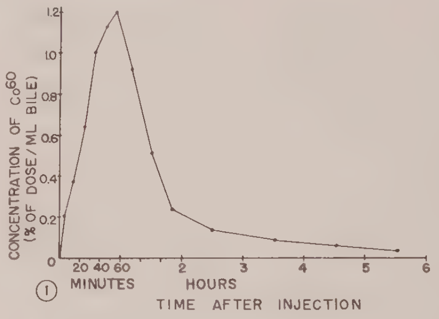
* Kindly supplied by Dr. L. Ellenbogen, Lederle Laboratories, Pearl River, N. Y.

Aliquots were dried in planchets and counted in a γ-scintillation counter with a flat crystal of NaI. Under the conditions used, 1 mμg of $Co^{60}B_{12}$ gave a count of about 80 above the background of 140 per minute. The binding powers of IF and bile specimens were determined after exhaustive dialysis against large volumes of distilled water for 48 hours, and the basal bile served as the blank. The details of the technique for B_{12} binding measurement and the exhaustive dialysis have been described(4). Some of the bile specimens containing radioactivity were also subjected to equilibrium dialysis. In this dialysis, 2 ml of bile in a Visking bag were dialyzed against 30 ml of distilled water under constant agitation for 48 hours, and both the inside and the outside of the bag measured for radioactivity.

Results. A mixture of 2 mg of IF and 500 mμg of $Co^{60}B_{12}$ was injected intravenously to the rabbit, and bile was collected every 10 minutes for the first hour, every 20 minutes for the next hour and hourly thereafter. The volume held by the cannula and the tubing was allowed to drain before actual timing was started. Concentrations of radioactivity measured in bile clearly demonstrated that excretion of Co^{60} began almost immediately following the injection and reached the peak between 50 and 60 minutes, decreasing sharply thereafter (Fig. 1).



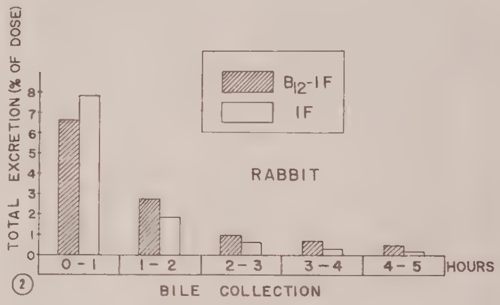


FIG. 1. Rate of Co^{60} excretion in bile following intravenous injection of IF—$Co^{60}B_{12}$ complex to the rabbit. A mixture of 2 mg of IF and 500 mμg of $Co^{60}B_{12}$ was administered. Figures are average of 2 rabbits.

FIG. 2. Total biliary excretion of IF and IF—B_{12} complex following intravenous administration to the rabbit. Shaded bars represent radioactivity excreted in bile following injection of a mixture of 2 mg of IF and 500 mμg of $Co^{60}B_{12}$; open bars represent B_{12} binding power excreted following injection of 2 mg of IF alone. Figures are average of 3 animals.

Under the same condition, bile was accumulated in hourly portions and subjected to exhaustive and equilibrium dialyses to determine the quantitative relationship between bound and free Co^{60}. The results (Table I) showed that most of the radioactivity in the 1st and the 2nd bile specimens remained in

TABLE I. Dialysability of radioactivity excreted in bile following intravenous injection of IF-$Co^{60}B_{12}$ to rabbit.

		Radioactivity (as mμg of $Co^{60}B_{12}$)			
		Exhaustive dialysis		After equilibrium dialysis*	
Bile specimen (Time of collection)	Vol. (ml)	Before (per ml bile)	After (per ml bile)	Inside (2 ml bile)	Outside
0-1 hr	9.5	3.7	3.6	7.3	0
1-2 "	7.5	2.0	2.0	4.1	0
2-3 "	6.4	.8	.8	1.5	0
3-4 "	6.9	.5	.4	1.0	0
4-5 "	5.8	.4	.4	.8	0

Two ml of bile used for dialysis, and duplicates were made wherever volume permitted.
* Inside—2 ml bile, outside—30 ml distilled water.

the cellophane bag after dialyses. Since the basal bile contained a B_{12} binding power equivalent to 1.5 mμg/ml, the undialyzed radioactivity in the 3rd, 4th and 5th specimens do not necessarily indicate the presence of an exogenous B_{12} binder. Thus, it occurred to us that Co^{60} excreted in the 1st and 2nd specimens might represent $Co^{60}B_{12}$ still bound to IF. The following experiment was carried out to clarify this point.

Four rabbits were divided into 2 groups, and a mixture of 2 mg of IF and 500 mμg of $Co^{60}B_{12}$ was injected intravenously to one group, and the same dose of IF without B_{12} to the other group. The bile was accumulated in hourly portions, and total radioactivity of each specimen was measured in the former and B_{12} binding power in the latter. The results (Fig. 2) clearly demonstrated that following intravenous administration of IF, there was a marked increase of B_{12} binding power in the bile during the first one hour. Noteworthy also was that the amount of binding power measured in the 1st one-hour specimen of the second group was very similar to the amount of radioactivity of the corresponding bile specimen of the 1st group, if both were expressed in terms of mμg of B_{12}. It seems likely therefore, that the B_{12} binding power excreted in the bile following intravenous administration of IF represented the original IF or IF in an altered form but still retaining the B_{12} binding property. It follows further that the radioactivity excreted in the 1st group also represented the original or a slightly modified IF-B_{12} complex.

A similar experiment was carried out with rats. Six adult female rats were divided into 2 groups, one group receiving intravenously a mixture of 1 mg of IF and 100 mμg of Co^{60}-B_{12}, the other group receiving the same dose of IF alone. Again, a significant amount of radioactivity or B_{12} binding power was measured in bile during the first hour. Furthermore, the quantitative relation between the radioactivity in bile of the 1st group and B_{12} binding power of the 2nd group was practically the same as that found with rabbits.

Discussion. The preferential hepatic uptake of intravenous IF-B_{12} complex first detailed by the author(4) may or may not be related to the natural function of IF. It can be argued that possible pinocytosis of IF molecules by the liver reticuloendothelial system may be responsible as is the case with antigenic heterologous proteins. Nevertheless, it is of interest to postulate the presence of a B_{12} carrier in the blood which is IF itself or behaves like it and has a strong affinity to the liver. As already pointed out, intravenously administered IF-B_{12} complex is not taken up by the spleen or any other organ which is rich in the reticuloendothelial system except the liver. The results of this study have provided further support to this hypothesis by demonstrating that both IF and IF-B_{12} complex are immediately excreted in bile without evidence of extensive chemical changes, following intravenous administration. If the uptake of IF or its B_{12} complex were executed by pinocytotic activity of the Kupffer cells, no explanation seems feasible for the demonstrated instantaneous transfer of the test material from the blood stream into bile, because of the anatomy of the lobule. It is only fair to presume that IF or IF-B_{12} is trapped in the Disse's space which is formed by the microvillous surface of the polygonal cells and the trabeculae of the Kupffer cells, and some of the trapped molecules immediately move from there into the bile canaliculi. The only possible route for such rapid excretion might be the electronmicroscopic gap between the polygonal cells which connects the Disse's space to the bile canaliculus(7). If all the IF molecules are to be engulfed by the Kupffer cells like certain colloid particles, it will take some time before they are ejected from the cells and excreted into bile. Inference may be made, therefore, that IF or IF-B_{12} complex has a strong affinity to the surface of the Disse's space. Such affinity may be due to the presence of a chemical acceptor on the cell surface. The hypothetical carrier of B_{12}, which was simulated by IF in this experiment, may be circulating in blood in minute quantities to regulate the hepatic uptake of B_{12}. A recent study by Brody *et al.*(8) of the kinetics of intravenously injected B_{12} in man has suggested a similar possibility.

Summary. Intrinsic factor or a mixture of IF and $Co^{60}B_{12}$ was injected intravenously to rabbits and rats in which a biliary fistula had been prepared, and excretion of B_{12} binding power or Co^{60} in bile was measured. It was

found that significant amounts of B_{12} binding power or Co^{60} appeared in the bile within one hour and that such excretion began almost immediately following intravenous administration. A possible mechanism of hepatic uptake and subsequent excretion into bile of intravenously administred IF or its complex has been discussed.

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Influence of Spermine and Related Substances on Susceptibility of *Bacillus anthracis* to Lysozyme.‡ (27052)

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In complex media certain strains of *Bacillus anthracis* become susceptible to the action of lysozyme only when bicarbonate is present during growth(1). This requirement for bicarbonate was not demonstrable in chemically defined media; under these conditions the strains remained susceptible to lysozyme when bicarbonate was omitted. Investigation of the cause of this difference revealed that the yeast extract present in complex media contained a dialyzable factor that caused susceptible strains to become resistant. It was the activity of this factor that was overcome by addition of bicarbonate to the growth medium(2).

Identification of the factor in yeast and elucidation of its action are of particular interest because of the possibility that related mechanisms may be involved in the bicarbonate requirements for elaboration of capsular polypeptide and protective antigen by this organism(3,4). The present report presents evidence that spermine, spermidine, and certain diamines reproduce the effect of yeast extract in inducing resistance to lysozyme.

Methods. The basal medium contained 2% Difco vitamin-free casamino acids, 0.04% Difco yeast extract, and 0.2% glucose. The small amount of yeast extract was sufficient to allow growth of the test organism in the absence of lysozyme but not in its presence. The medium was adjusted to pH 7.6 with sodium hydroxide and sterilized by filtration. Ultra-fine sintered glass filters were used for all sterilizing filtrations. Sterile sodium bicarbonate solution was added to the basal medium in the concentrations indicated.

The compounds tested were obtained from commercial sources, spermine and the diamines as hydrochlorides, spermidine as the phosphate, and agmatine and arcain as sulphates. They were dissolved in water and sterilized by filtration. Titrations were carried out in ¾″ x 6″ tubes provided with aluminum caps. Serial 2-fold dilutions of the substances tested were prepared in a volume of 0.5 ml. To each tube were added 0.5 ml of 1-1000 solution of crystalline lysozyme (Armour Laboratories) and 1.0 ml of basal medium. Controls without lysozyme, without test substance, and without inoculum were included in each titration.

The inoclum consisted of approximately 100 spores of strain B of *B. anthracis*, added

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as one drop of a suitably diluted stock suspension. Strain B is a typical lysozyme-susceptible culture(2). The tests were incubated at 37°, and growth was recorded after 40-48 hours.

Results. Various diamines, polyamines, and related compounds were titrated for their activity in permitting growth of the B strain in the presence of 1-4000 lysozyme. Titrations were carried out in the presence of 2 concentrations of sodium bicarbonate: $0.0005M$ and $0.005M$. In the complete absence of added bicarbonate irregular results were obtained, presumably because of variations in absorption of atmospheric carbon dioxide. Addition of $0.0005M$ sodium bicarbonate obviated this difficulty and allowed relatively reproducible titrations, although this low concentration doubtless reduced the activity of the compounds somewhat. The results are summarized in Table I.

TABLE I. Activity of Diamines and Polyamines in Allowing Growth of *B. anthracis* in Presence of 1-4000 Lysozyme.

Substance	Concentration allowing growth (μmoles per liter) $.0005M$ $NaHCO_3$	$.005M$ $NaHCO_3$
1-3 propanediamine	60	>2500
Putrescine	120	>2500
Cadaverine	600	>2500
Spermidine	6	> 500
Spermine	3	> 500
Agmatine	400	—
Arcain	>500	—

It may be noted that spermine and spermidine were effective in very low concentrations, 1-3 propanediamine and putrescine were also active but less so than the polyamines, and cadaverine was less active than putrescine. Agmatine, the monoguanidyl derivative of putrescine, was less active than putrescine itself, and arcain, the diguanidyl derivative, was inactive at the concentrations tested. The activity of all the compounds was markedly inhibited in presence of $0.005M$ sodium bicarbonate.

Discussion. The evidence suggests that spermine and spermidine are responsible for the activity of yeast extract in inducing lysozyme resistance in *B. anthracis.* These substances are active in very low concentration, and their effect, like that of yeast extract, is antagonized by bicarbonate. Spermine was isolated from yeast extract(5), and *Saccharomyces cerevisiae* was shown to synthesize spermine and spermidine(6). Furthermore, none of the numerous growth factors, amino acids, and other substances that were tested possessed similar activity(2).

Spermine and related substances are widely distributed in plant and animal tissues(7,8) and are essential or stimulatory factors for growth of *Hemophilus parainfluenzae*(9), *Neisseria perflava*(10), a mutant of *Aspergillus nidulans*(11), *Pasteurella tularensis*(12), and *Lactobacillus casei*(13). Putrescine, 1-3 propanediamine, spermine, and spermidine are accumulated or interconverted by a variety of microorganisms(6). Gram negative bacteria evidently accumulated larger amounts of putrescine than gram positive organisms(14). The synthesis of spermidine from putrescine and methionine in *E. coli* has been elucidated (15) and oxidative degradation of the polyamines by *Pseudomonas aeruginosa* and by *Neisseria perflava* has been investigated(16, 17).

Despite considerable knowledge of the biosynthesis, interconversion, and degradation of spermine and related compounds, their essential metabolic functions remain unknown. Accordingly, no explanation for their effect on resistance to lysozyme in *B. anthracis* can be postulated with assurance. With respect to the relative activities of the various compounds, the present system is analogous to growth stimulation of *P. tularensis* and *L. casei,* in that spermine and spermidine are significantly more active than the diamines. Recently it has been demonstrated that in *Micrococcus lysodeikticus,* resistance to lysozyme is associated with an increased concentration of O-acetyl groups in the cell wall(18). Should resistance in *B. anthracis* be explicable on a similar basis, tentative inferences could be made regarding the mode of action of spermine and related substances.

A ready explanation is not available for the activity of bicarbonate in overcoming the effect of spermine and related compounds. No similar effect of bicarbonate with respect to the other biological activities of these substances appears to have been recorded. In preliminary experiments, addition of bicarbonate to the growth medium did not suppress the accumulation of spermine-like activity in culture filtrates of *B. anthracis*. However, the possibility has not been excluded that the active substance was initially present in the culture as an inactive carbamyl compound, which was hydrolyzed during removal of bicarbonate prior to testing. Reaction of putrescine with carbamyl phosphate has been demonstrated in the presence of yeast and mold enzymes(19). It is perhaps significant that arcain, the diguanidyl derivative of putrescine, was inactive in the present system; dicarbamyl derivatives of the active compounds have not yet been tested.

Summary. Spermine was active at a concentration of 3 μ moles per liter in allowing growth of a lysozyme-susceptible strain of *B. anthracis* in the presence of 1-4000 lysozyme. Spermidine, 1-3 propanediamine, putrescine, and cadaverine were respectively 1/2, 1/20, 1/40 and 1/200 as active as spermine. The activity of all of the substances was overcome by addition of 0.005*M* sodium bicarbonate to the medium.

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Multiplication of Echo 9 Virus in Suspensions of Human Leucocytes.* (27053)

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Isolation of ECHO virus type 9 from the serum of 10 of 18 children(1) indicates that viremia occurs commonly in human infection with this agent. In attempting to investigate a possible role of leucocytes during ECHO 9 viremia, experiments were performed to determine whether human leucocytes *in vitro* were capable of supporting multiplication of the virus. A previous publication(2) reported the multiplication *in vitro* of measles virus in human and simian leucocytes.

Materials and methods. Human blood cell suspensions were prepared by a modification of a previously described method(2). Venous blood was withdrawn from a healthy donor whose serum neutralized 100 TCD_{50} of ECHO 9 virus in 1:16 final dilution. Heparinized blood was centrifuged at 1500 RPM for 10 minutes. Buffy coat was washed 3× and finally resuspended in medium 199(3) containing 20% calf serum, penicillin and streptomycin. Final medium was adjusted to pH 7.4

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with $NaHCO_3$. The final suspension, containing 5-14 × 10^6 nucleated cells/ml and an uncounted number of red cells, was delivered in 1 ml aliquots to 125 × 16 mm screw cap test tubes. *Virus.* ECHO 9 virus, Hill strain, was furnished by Dr. Herbert A. Wenner. A stock prepared in primary rhesus monkey kidney cell (MKC) cultures had an infectivity titer of 6.25 log $TCD_{50/0.1\ ml}$. *Viral Assay.* All infectivity titers were determined in MKC cultures, which were prepared and initially nourished in Hanks' balanced salt solution (BSS) containing 0.5% lactalbumin hydrolysate (LAH) and 2-10% calf serum. Prior to use cultures were refed with Eagle's basal medium(4,5) prepared with BSS. Penicillin, streptomycin and mycostatin were added to both media. Serial 10-fold dilutions of inoculated blood cell suspensions were prepared in BSS with 0.5% LAH and inoculated in 0.1 ml aliquots into 3 cultures per dilution. Cultures were kept stationary at 36-37°C, observed for cytopathic effect and discarded after 2 weeks. Titers were calculated by the Reed-Muench method and expressed as the log $TCD_{50/0.1\ ml}$ of inoculum.

Experimental. Propagation of ECHO 9 virus in the suspended blood cells was demonstrated in the following representative experiment (Table I). A 1 ml cell suspension inoculated with 0.1 ml of a 100-fold dilution of stock virus was incubated in a roller wheel at 35-36°C. At 2-3 day intervals 0.1 ml aliquots were transferred to fresh cell suspensions and at the same time the infectivity titer was determined for a similar aliquot. After 5 such subcultures, assuming no viral inactivation at 35-36°C over an 11 day period, total dilution alone would result in the extinction of non-multiplying virus in the original inoculum. However, at this stage the actual log infectivity titer was 4.25, demonstrating that viral propagation had occurred. It was then shown in 6 of 7 successive experiments that multiplication of virus occurred during single passage in blood cell suspensions. In contrast, neither medium 199 plus 20% calf serum without leucocytes, rapidly frozen and thawed leucocytes nor suspensions comprising only red blood cells supported ECHO 9 multiplication. This emphasizes the dependence of viral multiplication on the presence of intact leucocytes. In similar experiments the virus failed to multiply in rabbit blood cell suspensions during single or multiple passages.

TABLE I. Infectivity Titers of ECHO 9 Virus during 5 Serial Passages in Human Blood Cell Suspensions over an 11 Day Period.

Passage Level	Day of Exp.	Max. Infectivity Titers of Suspensions (log $TCD_{50/0.1\ ml}$)	
		Estimated*	Observed
0	0	3.25	—
1	2	2.25	1.25
2	4	1.25	2.25
3	7	.25	$>$2.25†
4	9	$<$ .25	3.50
5	11	$<$ .25	4.25

* Estimated titers, in absence of viral multiplication, are based on dilution and the assumption that there is no loss due to thermal inactivation. Zero day titer is the calculated infectivity titer of the original suspension immediately after inoculation with diluted stock virus. Titer of stock virus was 6.25 log $TCD_{50/0.1\ ml}$. All titers were determined in MKC cultures.

† Endpoint not reached at final dilution tested.

Experiments were performed to determine from which fraction of the blood cell suspension virus was recoverable. An inoculated suspension was centrifuged after 3 days incubation. Cell-free supernatant fluid yielded approximately 100-fold less infective virus than did whole blood cell suspensions when simultaneous assays were performed in MKC cultures. It was not determined whether virus associated with cells was primarily intracellular or surface attached.

After 2-3 days incubation neither infected nor non-infected leucocytes appeared to attach to glass and the majority of cells maintained their characteristic morphologic appearance when stained with Wright's or hematoxylin and eosin stains. The appearance of degenerating leucocytes in inoculated and uninoculated suspensions was similar. Mitotic cells were not observed. Study of numerous preparations stained with fluorescent antibody or acridine orange failed to indicate which cell of the leucocyte series supported viral multiplication.

Discussion. It was shown previously that human white blood cells *in vitro* supported propagation of measles virus(2). Demonstration of the ability of human leucocytes *in vitro* to support the growth of ECHO 9 virus provides one more example of the susceptibility of these cells to viral infection and reinforces the possibility that leucocytic infection may occur *in vivo;* further, it suggests that in studies of viremia leucocytes should not be excluded from specimens to be tested.

The observations with ECHO 9 and measles viruses do not justify the assumption that leucocytes are universally susceptible to viruses which are known to produce viremia. In the case of infection with type 1 poliovirus, viremia has been well documented(8). However, experiments similar to those now reported and conducted recently in this laboratory have not provided evidence that type 1 poliovirus multiplies in leucocytes.

Whereas leucopenia is associated with measles, in human infection due to ECHO 9 virus the peripheral white count is usually within normal limits or slightly diminished and may rarely be elevated early in the illness (6,7); consequently, although it is suggested that both viruses may infect leucocytes *in vivo*, it is not clear that such infection influences leucocyte survival.

Summary. Propagation of ECHO 9 virus in suspensions of human blood leucocytes has been described.

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Demonstration of Serological Specificity in the Reaction of Individual Gamma Globulins with Rheumatoid Factors by Hemagglutination.* (27054)

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Two general serological methods utilizing human gamma globulin for detection of rheumatoid factors in human serum are now being used. Human gamma globulin isolated by the Cohn alcohol fractionation method (Fraction II) from pooled plasma may be coated on latex particles(1) or may be attached to red blood cells by means of a tannic acid bond(2). Although the sensitivity of the several techniques utilizing pooled gamma globulin vary, they are all characterized by an absence of specificity for the several types of rheumatoid factor as defined by the second method in general use. The Gm system defines the rheumatoid factors by means of human Rh_0 positive red cells coated with selected incomplete anti-D sera(3). The rheumatoid factors are defined as anti-Gm factors of differing types depending on the Gm characteristics of the anti-D coat. Rheumatoid sera of the Gm (a+) type may be negative by this test or may show a marked prozone reaction. Harboe (4) has reported that such sera become positive and lose their prozone reaction when the euglobulin fraction is studied free of the coexisting specific inhibitor contained in the serum pseudoglobulin fraction.

Past efforts to utilize 7S gamma globulin isolated by physical and chemical means from individuals as a cell coating material to detect the several types of rheumatoid factors as defined by the Gm system have been unsuccessful (5,6,7). Isolated gamma globulin fractions from individuals have been either nonreactive or when partially denatured by heating or

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other means have shown a reactivity similar to that of pooled commercial gamma globulin in its non-specificity.

The present study was undertaken in an effort to utilize tannic acid-treated sheep erythrocytes coated with 7S gamma globulin obtained from normal and rheumatoid individuals to detect serologically distinguishable rheumatoid factors.

Material and methods. Rheumatoid sera: Its euglobulin and pseudoglobulin fractions. Sera were obtained from patients with classical peripheral rheumatoid arthritis (R-sera). Rheumatoid factor activity was measured by the tanned cell procedure† of Heller *et al.*(2) after heat inactivation of serum complement and absorption with washed sheep erythocytes. The euglobulin and pseudoglobulin‡ fractions of serum were obtained by the technique described by Ziff *et al.*(8) using dialysis against a M/150 phosphate citrate buffer pH 6.3. The precipitated euglobulin fraction after being washed in the same buffer was dissolved in a volume of pH 8.0 phosphate saline buffer equal to that of the original amount of serum used. Denatured material which would not dissolve was discarded.

Titers are expressed as the $\log_2$ of the highest dilution causing agglutination using a 1:28 initial dilution in a pH 8.0 phosphate saline buffer containing 1:2,000 bovine serum albumin.

Isolation of gamma globulin. One serum from a patient with peripheral rheumatoid arthritis (R4360) and 2 normal sera were used as the sources of gamma globulin. The 2 normal sera, kindly provided by Dr. Hugh Fudenberg, were N4226, 7S Gm type Gm 1(a+b+x−) Gm 2(a−b+) and N4227, 7S Gm type Gm 1(a−b+x−) Gm 2(a−b+). The 7S gamma globulin from individual sera was obtained in 2 chromatographic steps.

a) Gel filtration utilizing Sephadex G-25 (9,10) was used to separate proteins soluble in 0.004 M pH 8.0 phosphate saline (approximately 95% of all serum protein—designated peak I) from those gamma globulins not soluble at this low buffer electrolyte concentration.

b) The 7S gamma globulin was isolated from peak I by means of anionic exchange chromatography on diethylaminoethylecellulose (DEAE)§ as described previously(9).

Isolated protein was preserved by quick freezing in a dry ice-acetone bath. The 7S gamma globulin character of the isolated protein was established by analytic ultracentrifugation and immunoelectrophoresis against a horse antiserum to normal human serum(10).

Coating of cells. Washed sheep red cells were coated with the individual gamma globulins using the method described by Heller(2). A rabbit antiserum to pooled human 7S gamma globulin was used to establish that the cells were coated. The tannic acid-treated cells were exposed to a concentration of 1.0 mg of protein/ml.

Inhibition procedure. A 0.5 ml volume of dilutions of whole serum or its pseudoglobulin fraction was used to inhibit agglutination by 0.5 ml of a stated concentration of rheumatoid serum. The dilution of the agglutinating serum was added to the serial dilutions of the inhibiting material. The tubes were incubated at 37°C in a water bath for 1 hour and 0.5 ml of a 0.25% concentration of the coated cells then added. The rack was then maintained at 4°C overnight. Complete agglutination is designated as 2, indicating a flat blanket of cells, while total inhibition refers to a tight button of cells and is designated as zero.

Results. Cells coated with the 7S gamma globulin from R4360 as obtained from the chromatographic procedures and after different degrees of heating of the isolated globulin were tested against 3 R sera which showed strongly positive tests using cells coated with commercial pooled gamma globulin. The results shown in Table I indicate that all cells were about equally coated as indicated by the rabbit antiserum but that the strength of agglutination by R sera was much weaker with the unheated gamma globulin than with that heated to 48°C for 10 minutes. Heating gamma globulin to 63°C for 5 minutes causes loss of the specificity of the reactions as indicated by serum R4499 giving a pattern and end point identical with cells coated with pooled commercial gamma globulin. For these reasons all

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† Commercial pooled human globulin obtained through the courtesy of American National Red Cross, Washington, D. C.

‡ This fraction also contains the serum albumin.

§ Coarse mesh, 0.9 mEq/g, obtained from Brown & Co., New Hampshire.

TABLE I. Agglutination Pattern of R Sera Tested with Cells Coated with Unheated and Heated Gamma Globulin from R4360.

Serum R	γ-R4360* cell coating	Log₂ dilution of serum (Initial dilution—1:28)							
		1	2	3	4	5	6	7	8
4501	Unheated	0	0	1	1	1	1	0	0
	48°C-10 min	2	2	2	2	2	2	1	0
	63°C- 5 "	2	2	2	2	2	2	2	0
2285	Unheated	0	0	0	1	1	1	0	0
	48°C-10 min	1	1	2	2	2	2	0	0
	63°C- 5 "	2	2	2	2	2	2	2	2
4499	Unheated	0	0	0	0	0	0	0	0
	48°C-10 min	0	0	0	0	0	0	0	0
	63°C- 5 "	2	2	2	2	2	2	1	0

* Log₂ titer using rabbit antiserum to human gamma globulin.

Cells coated with γ unheated —5.
" " " γ heated to 48°C-10 min—6.
" " " γ heated to 63°C- 5 min—6.

cells used in the following studies are coated with protein solutions after heating the protein in a concentration of 1 mg of protein/ml to 48°C for 10 minutes. No grossly detectable clouding of the solution is produced by this degree of heating.

TABLE II. Agglutination of Cells Coated with Individual Gamma Globulins Tested Against 5 R Sera and their Euglobulin Fractions.

Serum R		Log₂ titer of cells coated with:			
		Pooled γ	γ-R4360	γ-N4226	γ-N4227
4501	Whole serum	7	7	6	0
	Euglobulin	7	9	8	0
4405	Whole serum	8	0	0	0
	Euglobulin	12	0	0	0
2285	Whole serum	8	6	7	0
	Euglobulin	8	7	8	9
4373	Whole serum	9	0	0	0
	Euglobulin	12	6	12	0
4647	Whole serum	8	6	0	0
	Euglobulin	10	8	7	0
Rabbit antiserum		5	6	8	6

Table II lists the titer of 5 whole R sera and their euglobulin fractions expressed as the $\log_2$ of the dilution at the end point. All positive tubes showed a maximum agglutination pattern. The 5 sera used all showed about the same end point to cells coated with pooled gamma globulin and showed the same or higher titers when their euglobulin fraction was tested with these cells. The results using the cells coated with individual gamma globulins show that no individual coat duplicates the coating of pooled gamma globulin. Serum R2285 is the only one reacting as the euglobulin to all coats while the other 4 each have one or more instances of a totally negative test when using euglobin. Serum R4405 lacks rheumatoid factor for all of the individual gamma globulins. The presence of a specificity of the 3 individual coats is shown both in the results using whole serum as well as the euglobulin fractions. Serum R2285 is the only one in this panel having rheumatoid factor reactive with the determinants of γ-N4227 and this does not appear related to the euglobulin of highest reactivity with pooled gamma globulin coated cells.

Three R sera having strongly positive tests to pooled gamma coated cells but no titer to cells coated with γ-N4226 were selected to test the inhibiting capacity of R sera and its pseudoglobulin fraction against serum R4501 which aggultinates γ-N4226 coated cells. Table IIIa shows that 2 of these sera contain rheumatoid factor elements in their euglobulin capable of reacting with γ-N4226 coated cells. The pseudoglobulin fractions show no reactivity.

TABLE III. Inhibition of Rheumatoid Serum.

a) Characteristics of inhibiting sera

Serum R		Log₂ titer cells coated with γ-N4226
	Whole serum	0
4647	Euglobulin	7
	Pseudoglobulin	0
	Whole serum	0
4405	Euglobulin	0
	Pseudoglobulin	0
	Whole serum	0
4373	Euglobulin	12
	Pseudoglobulin	0

b)

Inhibiting material		Log₂ dilution of inhibiting material*†‡ (Initial dilution 1:100)													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
R4647	Whole serum	1	2	2	2	2	2	2	2	2	2	2	2	2	2
	Pseudoglobulin	1	2	2	2	2	2	2	2	2	2	2	2	2	2
R4405	Whole serum	0	0	0	0	0	0	0	0	0	0	1	1	2	2
	Pseudoglobulin	0	0	0	0	0	0	0	0	0	0	1	2	2	2
R4373	Whole serum	0	0	0	0	0	0	0	0	0	1	1	1	2	2
	Pseudoglobulin	0	0	0	0	0	0	0	0	0	1	2	2	2	2

* Cells coated with γ-N4226.

† Agglutinating serum R4501—0.5 ml of 1:56 dilution in each tube.

‡ Control: .5 ml of 1:56 dilution of R4501;
.5 ml of diluent;
.5 ml of γ-N4226 coated cells.
Result: 2+ agglutination.

The dilution of R4501 to be inhibited (1:56) provides a control tube showing maximum agglutination using 0.5 ml of buffer in place of the dilution of inhibiting serum. Serial dilution of the inhibiting materials were all started at a 1:100 dilution relative to the whole serum. Table IIIb shows that a striking difference in inhibitory capacity exists for serum R4647 which shows little or no inhibition ability compared to sera R4405 and R4373. The inhibitory capacity of the pseudoglobulin fractions parallels those of the whole sera. All 3 of the sera examined for inhibitory capacity showed moderately elevated serum gamma globulin on paper electrophoresis.

Discussion. Although the 7S gamma globulin isolated chromatographically from individual normal and rheumatoid sera shows the ability to coat tannic sheep erythrocytes as indicated by a rabbit antiserum, the pattern of agglutination of such coated cells tested against rheumatoid serum was extremely difficult to read. Prior studies employing chemical and heating methods for the aggregation of gamma globulin have shown increased reactivity after such treatment as judged by the magnitude of the precipitation reaction between gamma globulin and rheumatoid sera(5,11,12). No direct investigation of the aggregate content of the chromatographically isolated gamma globulin was made in the present study. Our objective was to obtain preparations of gamma globulin from individual sera which would exhibit a spectrum of reactivity to different rheumatoid sera similar to that observed in the Gm system utilizing select anti-D sera to coat human Rh(+) cells. Serum R4499 proved a valuable reagent since it agglutinated cells coated with pooled gamma globulin yet failed to agglutinate cells coated with γ-R4360. Heating γ-R4360 to 48°C for 10 minutes provided a coating material which gave a maximum agglutination pattern with R sera No. 4501 and R2285 yet showed no reaction with R4499. This differential reactivity was lost by heating γ-R4360 to 63°C for 5 minutes prior to coating cells (Table I). If pooled gamma globulin contains all possible determinant groupings and this accounts for its reactivity with all R sera, it is difficult to conceive how heat can impart such heterogeneity to the gamma globulin of a single individual which demonstrates a high order of selectivity after lesser degrees of heating. A possible explanation may lie in the exposure of a portion of the gamma globulin molecule which reacts with different rheumatoid factors and itself lacks determinant specificity. Aliquots of these same samples of individual gamma globulins which were frozen and thawed several times or permitted to remain at room temperature or at 37°C for several hours showed the same augmentation of reactivity as produced by heating to 48°C for 10 minutes. This latter procedure would appear to provide a more reproducible reagent and is used throughout the present investigation.

The results shown in Table II indicate that the totality of reactivity found in the pooled gamma globulin coated cells is not found in any of the 3 individual gamma globulins. Three types of reaction patterns are noted in this study. In those sera which show strong reactivity to an individual gamma globulin coat in both the whole serum and its euglobulin fraction, the pseudoglobulin of that serum is presumed to be deficient in the inhibitor specific for the rheumatoid factor of the serum which is reacting with an individual gamma globulin coated cell. The frequent observation of higher titers for the euglobulin fraction compared to the whole serum would suggest this is a quantitative rather than qualitative lack. This does not imply that the serum is deficient in inhibitors complementary to other rheumatoid factor specificities. This is demonstrated in the reaction patterns of sera R2285 and 4647. The presence of strong agglutination ability by the euglobulin with none demonstrable by the whole serum is taken to indicate an amount of inhibitor in the pseudoglobulin at least sufficient to react with all of that rheumatoid factor specificity present. Finally, the absence of agglutinating ability in both the whole serum and its euglobulin fraction for a cell coated with an individual gamma globulin indicates the absence of rheumatoid factor capable of reacting with that particular cell coating. From the results shown in Table II, γ-R4360 would appear more like γ-N4226 than γ-N4227. Rheumatoid sera having rheumatoid factor specificity complementary to the determinant groups of γ-N4227 would appear to be uncommon compared to those reactive with γ-R4360 and γ-N4226.

Based on the reasoning presented for the direct agglutination results, 2 of the 3 sera selected to study inhibitory capacity against R4501 (Table IIIa,b) show inhibitory capacity. The 3rd serum R4405 lacking reactive rheumatoid factor for γ-N4226 used as a coating material cannot be shown to have inhibitory activity for the specificity of this test system by direct agglutination.

The results shown in Table IIIb indicate that serum R4405, although lacking rheumatoid factor activity for cells coated for γ-N4226 does contain significant amounts of an inhibitor for the γ-N4226 versus serum R4501 test system. Although sera R4647 and R4373 show a qualitatively similar reaction pattern in Table IIIa they show a completely opposite inhibitory capacity in Table IIIb. The inhibitory capacity of the pseudoglobulin fractions of these sera were examined, to rule out the possibility that the lack of inhibitory capacity in serum R4647 is due to an exact balance of inhibitory material and rheumatoid factor in the whole serum. A possible explanation for the behavior of these 3 sera studied for inhibitory capacity based on multiple specificities for both inhibitory activity and rheumatoid factor activity is shown in Table IV.

TABLE IV. Possible Mechanism of Inhibition and Non-Inhibition.

	System to be inhibited	
	Cell coat γ-N4226	Agglutinating serum R4501
	1, 2	Anti 1
	Specificity of inhibiting sera	
	Inhibition specificity	Rheumatoid factor specificity for cell coating γ-N4226
Inhibiting serum R4405	1	Lacks anti 1 & anti 2
" " R4373	1	Anti 1
Non-inhibiting serum R4647	2	" 2

The ability of serum R4647 to show slight inhibitory ability is reminiscent of the findings of Harboe(13) that anti-Gm(a) and anti-Gm(x) inhibiting substances can be found in small amounts in Gm(a− x−) sera.

Whether or not the specificity of gamma globulin obtained from individuals when coated on tannic acid-treated red cells parallels that reported utilizing the sensitized human Rh(+) cell system cannot be stated. Some type of specificity is apparent both by direct agglutination and by inhibition. This technique should permit classification of 7S gamma globulin types in both normal and rheumatoid individuals as well as classification of different types of rheumatoid factors without recourse to rare anti-D cell typing sera. The presence of several types of rheumatoid factor in the same serum has been noted previously by chemical fractionation(14,15) as well as by Gm typing (16,17,18,19). The phenomenon of coexisting inhibitory material with rheumatoid factor in the same serum as noted in the Gm system especially in Gm(a+) individuals(4) seems also to apply to the present system.

Summary. Gamma globulin (7S) isolated by chromatographic techniques from 2 normal individuals and from one patient with rheumatoid arthritis coats tannic acid-treated sheep erythrocytes and detects rheumatoid factors with a specificity not found using pooled human gamma globulin. The serological specificity of individual gamma globulin preparations is destroyed by heating the protein to 63°C for 5 minutes. The specificity is also observed in an inhibition system using whole rheumatoid serum or its pseudoglobulin fraction. The results may be explainable by the presence of multiple determinants in both the 7S gamma globulin isolated from individuals as well as in the rheumatoid factor complex found in individual rheumatoid sera.

The author expresses his appreciation for technical assistance of Miss Mary Patricia Schaefer.

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Interactions of the Oral Microbiota
I. A System for the Defined Study of Mixed Cultures.* (27055)

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The net effect of a mixed microbial population is often greater or smaller than the simple sum of activities of the individual species growing in pure culture. It is therefore not possible to predict with certainty the influence of a single species, whose biochemical and growth characteristics have been determined solely in pure culture, on one or more different species. To the best of our knowledge there are no satisfactory techniques which allow measurement of interactions between 2 or more species of microorganisms growing in a dynamic state similar to that of nature and which at the same time permit control over basic factors of growth and population.

Until recently, attempts to assess bacterial interactions *in vitro* have fallen into 2 groups: a) growth in closed systems with selective isolation of components at varying intervals as reported by Annear(1) among others, and b) effect of metabolites produced by one species on another, the organisms being separated by a permeable membrane or similar barrier as described by Wilson and Goaz(8). A quantitative aspect was approached by Rosebury(7) with his relatively simple "drop pair" technique in which each of 2 different species diluted over a one-thousand-fold gradient are cultured as overlapping pairs on agar plates. While this technique can be evaluated mathematically, it is restricted to 2 species at a time and yields little information on products formed.

A limiting aspect of these methods (closed systems) is the lack of control over growth dynamics. Continuous culture permits controlling this factor and was suggested for early mixed populations by Rogers and Whittier(6). More recently, Zybrycki and Spaulding(9) constructed a simple continuous system for mixed cultures but cited no data on the interactions observed. A more sophisticated concept of continuous culture stems from the contribution of the chemostat by Novick and Szilard (5). The basic premise of this development assumes that multiplication of organisms in the growth cell is directly proportional to the rate at which some critical limiting nutrient is admitted to this cell. Novick(4) has also discussed the growth of pure cultures in a 2-stage arrangement in which the output of a chemostat is fed to a second growth cell. If nutrients in the second system are not limited, it becomes possible to define mathematically the steady state growth which results so that:†

$$X_2^\circ k^\circ m - F_1 + F_2/V_2 + \frac{F_1}{V_2} X_1^\circ = 0$$

In which: X_1° and X_2° respectively are the bacterial populations entering and leaving the

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† Mathematical symbols employed are those of the American Standards Assn. as cited by Gerhardt and Bartlett(3).

growth cell. k°m is maximum growth rate constant obtained from log phase of the organisms in pure culture. F_1 and F_2 are flow rates to the growth cells and V is cell volume.

Our study represents an extension of this 2-stage arrangement to permit application of previously recognized bacterial growth principles to the study of mixtures of bacteria growing under continuous and defined conditions.

Materials and Methods. To develop the desired system it seemed reasonable to experiment with a pair of organisms present in high levels in the mouth, one of which was known to furnish a metabolite utilizable by the other. The oral flora have an excellent example of this relationship in *Streptococcus salivarius* and *Veillonella alcalescens.* The former provides lactic acid from carbohydrates; the latter utilizes lactate but not the carbohydrate. Therefore, *S. salivarius* and *V. alcalescens* were grown in pure culture in individual chemostats and then combined for mixed growth at a second stage.

The chemostat cells were constructed with flat bottoms to permit the use of magnetic stirring bars. Each growth cell was equipped with additional standard taper ports to permit sampling and continuous measurement. The medium for each chemostat was metered by a peristaltic pump from an individual reservoir to an 0.0055 in. orifice at the chemostat. This size was necessary to assure constant feed rates of 8-11 ml media/hr to an 80 ml cell so that retention time in the cell was 7-10 hours. Other retention times might be used if physiologically young or physiologically aged cells were desired. Volumetric flow through each of the systems was carefully measured by calibration of the drop rate through the orifice. On the basis of experience over a number of runs, it was noted that the actual volumes deviated less than 2% from those calculated by the drop rate. The growth cell for the mixed cultures was similar in construction to the others (chemostats) but the nutrient supply to this cell was such that minimum ratio of feed rate to growth cell volume (dilution rate) exceeded the value of the maximum growth rate constant (k°m) of the faster growing species. This was a critical requirement and only in this manner could a mathematically defined maximum rate steady state be obtained in the mixed (second) system. The flow of the entire system is diagrammed in Fig. 1.

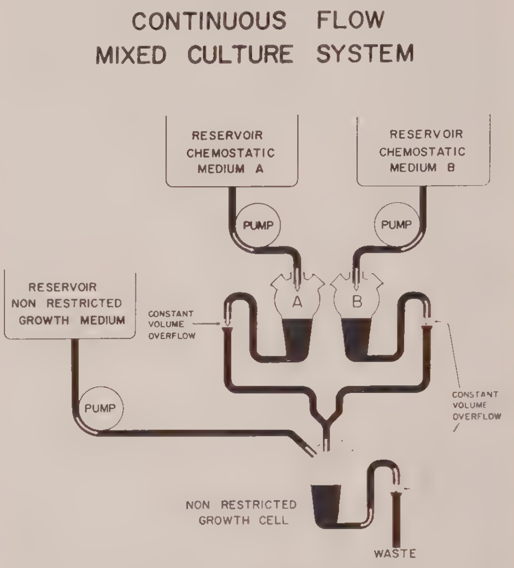
CONTINUOUS FLOW
MIXED CULTURE SYSTEM
RESERVOIR CHEMOSTATIC MEDIUM A
RESERVOIR CHEMOSTATIC MEDIUM B
PUMP
PUMP
RESERVOIR NON RESTRICTED GROWTH MEDIUM
CONSTANT VOLUME OVERFLOW
A
B
CONSTANT VOLUME OVERFLOW
PUMP
NON RESTRICTED GROWTH CELL
WASTE

FIG. 1. Flow diagram of 2-stage mixed culture system

The basic medium, consisting of yeast extract 0.2%, tryptone 0.2%, potassium phosphate (dibasic) 0.1%, sodium bicarbonate 0.1%, sodium thioglycollate 0.02%, was adjusted to pH 7.2. The limiting substrate for *S. salivarius* was 0.01% sucrose. For *V. alcalescens* the limiting substrate was 0.01% lactate. The same basic medium which was used for mixed growth contained 0.50% sucrose and 0.50% lactate and was adjusted to pH 7.2. The pH did not go below pH 6.8 at any time during growth. Temperatures of all growth cells were controlled in thermostatically regulated glycol baths. A magnetic motor below each bath actuated a stirring bar in both the chemostat and the glycol baths to insure uniform temperature, nutrient mixture, and bacterial densities. Populations of each species were determined by viable cell counts over several intervals for replicate results. Results reported are the product of at least duplicate runs. Media highly differential for the opposite species were used for mixed growth counts. The medium routinely used for counting *S. salivarius* was 5% sucrose agar with aerobic incubation. Veillonella were cultured anaerobically under an atmosphere of 10% carbon dioxide and 90% hydrogen on Douglas agar(2). Although *V. alcalescens* is an obligate anaerobe, growth of this organism in fresh media in the deep tube of the chemostat was equal to the growth rate obtained in a closed anaerobic tube. Therefore, it was not considered necessary continuously to purge the system with an inert gas.

Results. Generation times for each of the organisms growing in the second stage cell were calculated from the continuous culture growth rate equation (3):

$$FX_o - kXV = FX - Vdx/d\theta$$

in which k is related to generation time (θg) by the equation $\ln 2/\theta g = k$

These times obtained for both pure and mixed cultures of *S. salivarius* and *V. alcalescens* are given in Table 1.

The generation time for each species studied was markedly influenced by the presence of growing cells of the opposite member of the pair (Table 1). For example, in the steady state (where k°_m is less than F/V) generation time of *S. salivarius* was increased from 12 to 42 minutes in the presence of roughly 5.0×10^6 cells/ml and to 48 minutes with 1.4×10^7 cells/ml of *V. alcalescens* in the system. In the reverse situation, the normal generation time of *V. alcalescens* was between 10-12 minutes, whether in a bottle (log phase) or in the continuous culture cell but was lengthened to 15-21 minutes in the presence of $1.7\text{-}2.6 \times 10^6$ cells/ml of *S. salivarius.*

When grown under non-steady state conditions (F/V less than k°_m or 0.2 in Table 1.) tremendous increases were recorded for generation times of both species. Their extension to approximately 100 minutes was remarkably constant over the last 45 hours of a 70 hour run.

Discussion. Previous studies on mixed populations have employed techniques designed to demonstrate inhibition or stimulation of one microorganism by another, growing in close association with it in tubes or bottles or on agar plates. Such procedures are limited by providing an environment that is continuously and uncontrollably changing over the time that population density undergoes a several thousand-fold increase and average age of cells changes from youth to senescence. Such patterns defy definition and do not seem representative of the indigenous microbial systems existing in the body.

TABLE I. Generation times of *S. salivarius* and *V. alcalescens* grown as pure cultures and in mixture in a 2-stage continuous culture system.

		S. salivarius		*V. alcalescens*	
System type	Flow rate (F/V)	Generation time	Population	Generation time	Population
		min	cells/ml	min	cells/ml
Pure cult.	3.8	12	—	10-12	—
Mixed	3.9	42	1.8×10^6	21	4.7×10^6
"	3.8	48	2.6×10^6	15.5	14.3×10^6
"	3.9	44	1.7×10^6	17	10.5×10^6
"	0.2	101	9.35×10^7	100	14×10^6

The 2-stage continuous flow method described has obvious advantages for evaluation of interactions between 2 or more species of microorganisms because it is possible to regulate individually the ratio and levels of population for each species in the mixture by the following means: a) variation of levels of substrate furnished in the first stage chemostats, b) control of flow rate of medium to all growth cells, and c) control of flow rate of medium and number of organisms from each of the pure culture chemostats into the mixture.

Results obtained with *S. salivarius* and *V. alcalescens* demonstrate a distinct interaction between these species. The increase in generation times would not appear to be the result of depletion of critical nutrients since the medium used supported pure culture of either species at populations of at least 10^8 cells without noticeable change in generation time. This interaction is obviously subtle in nature and normally might not be detectable except by highly sensitive methodology such as we have described. Furthermore, the data can be expressed in generation time, which is a precise term with definite meaning to other investigators.

The observations reported here were obtained from non-defined reasonably complete bacteriological broth. Use of defined substrates on the one hand or use of tissue fluids on the other might well lead to different results. With proper selection of media, it should be practicable to investigate the influence of other substrates on mixed culture systems and to note the appearance of biochemical structures not present in the pure cultures feeding into the mixture.

Summary. A continuous 2-stage system for investigations of interactions between 2 or more species of bacteria is described. The system employs 2 or more chemostatically limited stages of pure cultures feeding to a non-limited mixed continuous growth cell. This permits comparison at any point on the growth curve of numbers and products of organisms in pure culture with numbers and metabolic products resulting from interactions between the same organisms in growing in mixed culture. The systems are mathematically defined and the data can be expressed in generation time. Results obtained with a mixture of *S. salivarius* and *V. alcalescens* indicate a distinct interaction betwen the strains employed. Generation time of each of these species was noticeably increased while growing in context with the other.

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Liver Haemodynamics and Age.* (27056)

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Marked variations in the pattern of liver haemodynamics have been observed in normal rats of different weight and age, maintained under identical conditions.† When related to the metabolic processes of the liver and its capacity to react to various agents, these changes may be of considerable importance.

In the present communication, data are presented on the haemodynamic pattern of the liver of the rat in different age groups.

Technique and Materials. Three groups of white male rats of the Hebrew University strain maintained under identical conditions and fed a standard diet were used. The first group (20 animals) weighed between 80 to 150 g, the second group (100 animals) weighed between 200 and 300 g, and the third group (20 animals) weighed between 300 and 450 g. The parameters examined were hepatic blood flow per gram liver tissue and per 100 g body weight, vascular space of the liver and portal vein pressure values.

Hepatic Blood Flow Determination. Hepatic blood flow was estimated as rate of removal of radioactive colloid from the peripheral blood by the reticulo-endothelial system of the liver. Heat denaturated iodine^{131}-labeled, human serum albumin was used as described by Benacerraf *et al.*(1). The dose of radioactivity injected varied from 0.01-0.1 μc per 100 g body weight and contained 0.05 mg or less of iodinated albumin per ml of water solution. The radioactive albumin was injected into the vena dorsalis penis and samples of 0.2 ml of blood were taken from the tail at 1, 2 and 3 minute intervals. The gamma radioactivity of the blood samples was measured in an "Atomic" scintillation counter.

Liver blood flow was calculated by multi-

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† Unpublished observations.

plying the constant rate of liver clearance by the distribution of radioactivity. The results were corrected by the efficiency factor of every group of animals. This factor was determined by injecting directly into the portal vein 0.2 ml of an albumin solution containing 1/10 of the amount of radioactive protein used for blood flow determination. During the next 5-10 seconds the portal and hepatic veins were ligated and cut, the liver excised and total organ radioactivity determined. Liver clearance efficiency was calculated as the ratio between organ radioactivity found and amount of radioactivity originally injected.

The amount of radioactivity cleared by the liver (distribution) was determined by injecting into the vena dorsalis penis an identical quantity of radioactive albumin as was used for blood flow determination. After 6 minutes, the time needed by the normal liver to clear the entire amount of radioactive material from the peripheral blood, the animals were sacrificed, the liver excised and amount of radioactivity in the organ measured.

Distribution of radioactivity outside the liver was measured in the thyroid gland, lung, spleen, kidney, bone marrow and lymph nodes.

Vascular Space Determination. The vascular space was determined as described by Klein(2) and estimated as the amount of hemoglobin present in a gram of liver. The left paramedial lobe was always used after ligation of the afferent and efferent vessels, special care being taken not to cause tissue injury and blood loss. Amount of hemoglobin was determined as cyanmethemoglobin measured in a Klett Summerson electrophotometer, and vascular space calculated as the ratio between hepatic and peripheral blood hemoglobin.

Portal Blood Pressure. Portal vein pressures were measured by a water manometer. The needle was inserted directly in the portal vein or in one of the branches of the mesenteric vein. Zero pressure was based on the anatomical level of the portal vein.

Results. The liver clearance efficiency in the young group of animals was 80% while in the older groups 90% of the injected radioactive albumin was cleared during one passage through the liver. Distribution of radioactivity in the liver of the younger group of animals was not significantly different from that found in the older group and varied between 72% to 74% of the amount injected. No difference in amount of radioactivity was found in the various liver lobes or in different areas of the same lobe.

Hepatic Bloodflow. When calculated as ml/min/100 g body weight, hepatic blood flow was 8.52 ± 0.73 in the younger group; 6.62 ± 0.9 in the middle age group and 3.49 ± 0.49 in the old age group (Table I). When calculated per g of liver tissue, blood flow was 1.90 ± 0.16 in the young age group, 0.92 ± 0.16 in the middle age group and 0.99 ± 0.13 in the old age group. This parameter indicates that the older the animal the smaller the amount of blood flow per gram liver tissue as well as per 100 g/BW.

Vascular Space. The highest vascular space values were obtained in the middle age group, the smallest in the younger age group and somewhat higher values were obtained in the

TABLE I. Liver Haemodynamics in Rats of Different Age Groups.

Bodywt	120g-150g	200g-300g	>300g
Liverwt g/100g bodywt	4.07 ± .10 * (21) †	3.47 ± .04 (107)	3.33 ± .09 (12)
Portal Pressure units saline	66 ± 2 (17)	70 ± 2 (78)	71 ± 9 (12)
Vascular Space ml/g	.108 ± .006 (20)	.148 ± .004 (103)	.128 ± .010 (20)
Hepatic Blood Flow ml/min/100g bodywt	8.52 ± .73 (13)	6.62 ± .19 (85)	3.49 ± .49 (12)
Hepatic Blood Flow ml/min/g liver	1.90 ± .16 (13)	1.92 ± .16 (85)	.99 ± .13 (11)

* M ± S.E. † Figures in parentheses indicate the number of animals.

older age group. Vascular space in the young group was 0.108 ± 0.056, in the middle age group 0.148 ± 0.004, and in the older age group 0.128 ± 0.010. The P value of the last 2 groups was 0.05.

Portal Pressure. Portal pressure values were constant and did not show significant changes when related to age, although there was a slight tendency towards higher values in the older animals. Portal pressure values varied between 66 mm saline in the young group and 70 mm saline in the middle age groups.

Discussion. When related to age, the most pronounced changes in the pattern of liver haemodynamics were observed in the parameters related to intrahepatic circulation. Low vascular space values associated with the highest rate of liver blood flow were characteristic of young animals. Decrease in blood flow concomitant with an increase in vascular space was found in the middle age group, and a marked reduction in liver blood flow was consistently observed in the older animals. The parameters most affected were the liver blood flow per gram liver tissue and per 100 g body weight, both of which showed a gradual decrease as the weight and age of the animal increased. Portal vein blood pressure showed only slight variations in the different age groups.

The low vascular space values found in the younger group indicate that either vascularization of the liver is not sufficiently developed or that the functional pathways are shorter and centrally situated. This would agree with observations already reported on the liver circulation of the rat examined by serial X-ray films(3). The distribution of the vascular pathways suggests that the liver lobules are not being equally perfused and that conditions of tissue anemia prevail in the peripheral areas. Tissue anemia at an age when liver growth is still active may be of considerable importance when related to growth, repair and capacity of the organ to react to various injurious agents(4,5,6). The high liver blood flow found in this group, associated with the lowest portal pressures and vascular space values, indicates that flow-pressure and flow-vascular space relationships are different from those postulated by Poisseuille for pure liquids. The liver blood flow at this age is probably not dependent on the cross-sectional area of the vascular bed or on levels of portal vein blood pressures. In contrast, liver blood flow per gram liver tissue remained unchanged in the middle age group, although vascular space was increased and portal pressure values remained constant as compared to the younger group. More striking was the 60% decrease in liver blood flow associated with unchanged values in the rest of the parameters, found in the older animals. These data indicate that increased or decreased blood flow rates are possible when other haemodynamic parameters remain constant, and, conversely, variations in portal pressure and vascular space are compatible with constant liver blood flow values. In addition, variations in portal blood pressure do not always reflect changes in vascular space values or in rate of hepatic flow. This confirms previous reports on changed relationships between the parameters controlling blood flow(7) and suggests that age may be one of the factors which influence the pattern of liver circulation.

Summary. Variations in the pattern of liver circulation in the rat have been observed in different age groups. Hepatic blood flow decreased as age of the animal increased and was not dependent on vascular space and portal blood pressure. Vascular space values were higher in the middle age group but portal vein blood pressures were unaffected by variations in age.

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Factors Influencing the Infectivity of Poliovirus Ribonucleic Acid.* (27057)

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III. *Role of Host Cell in Determining Infectivity of Ribonucleic Acid Under Varying Conditions.* The nature of the change responsible for loss of infectivity of free poliovirus ribonucleic acid (RNA) in the presence of isotonic salt solution and complete recovery when salt concentration is increased(1) is of interest in a number of disciplines. Fraenkel-Conrat(2), Doty and others(3) have reported that a structural change in the RNA molecule occurs with changes in salt concentration. This paper presents data which indicate that modification of the host cell also occurs with changes in salt concentration. Further, host cell susceptibility to RNA infectivity varies with increasing sucrose concentration, age of cell, nutrition factors during growth and temperature during invasion by RNA.

Materials and Methods. Cell lines. Amnion cells (Fernandes line(4)), HeLa cells (a cloned line selected in this laboratory for susceptibility to polioviruses(5)), and HEP_2 cells were grown for a week in 32 oz prescription bottles, then were treated with trypsin and transferred to 60 mm Petri dishes for growth of monolayers.

Media. Unless otherwise stated, all cell lines were grown in bottles and plates in a modified Eagle's solution made up with double the usual amounts of amino acids and vitamins.

RNA. RNA was prepared by phenol treatment of type 1 poliovirus (Mahoney strain) grown in tissue culture(6,7).

Inoculation of monolayers for measuring degree of infectivity by plaque counts. Before inoculation, cells were washed twice with isotonic salt solution buffered with Tris to pH 8. The inoculum of 0.1 ml of RNA preparation was left on the plates for 30 minutes at room temperature before nutrient agar layering, unless the routine was varied, as stated in the text. In some instances, where the inoculum was considered traumatic or adsorption time was long, the cells were washed once with isotonic salt solution before layering with nutrient agar. Details of media and technique were described previously(7).

Adsorption time. The term "adsorption time" is used in this paper to indicate the interval between inoculation of RNA and layering with agar or serum-containing solution to inactivate free RNA.

Inactivation of RNA and whole virus. In experiments measuring adsorption time, RNA was inactivated at the time indicated by layering the plates with 2 ml of buffered isotonic salt containing 10% horse serum or normal monkey serum. Whole virus was inactivated similarly with a solution containing 10% anti-type 1 polio monkey serum.

Experimental Results. The Salt Effect. Influence of salt concentration on infectivity of polio RNA in 3 cell lines. To differentiate the effect of salt on the RNA molecule from the effect of salt on the host cell, infectivity of the same RNA preparation was tested simultaneously in amnion, HeLa and HEP_2 cells. The cells in monolayers were inoculated with RNA diluted in the concentrations of NaCl and KCl noted in Fig. 1, and susceptibility was determined by counting the number of plaques which appeared under the conditions described. Fig. 1, presenting composite results from 5 experiments, indicates that amnion and HeLa cells are similar in relative susceptibility to RNA suspended in different concentrations of NaCl and KCl. Uniformly, sodium ions are more effective in enhancing infectivity at lower molarities than potassium ions. The 2 lines respond quite differently, however, to RNA diluted in increasing concentrations of both salts. HeLa cells are more susceptible to RNA at salt concentrations below 1 M than amnion cells, but titer is little enhanced with increase in salt concentration beyond this point. On the other hand, amnion cell susceptibility to RNA infection is roughly proportional to the salt concentration of either diluent until the limit of cell tolerance for salt

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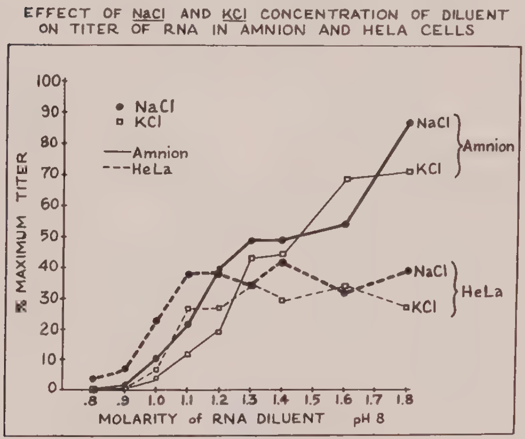
* Aided by a grant from The National Foundation.

is reached at 1.8 to 2 M. Subsequent tests with HEP_2 cells carried out with NaCl alone indicate that HEP_2 cells are the most sensitive of the 3 to the influence of this salt. RNA infectivity titers in HEP_2 cells at low molarities are higher than those of HeLa cells, and at higher molarities there is a titer increase proportional to increase in salt concentration similar to that in amnion cells but the increments continue at a higher level.



Points on graph represent average of values from 5 experiments.

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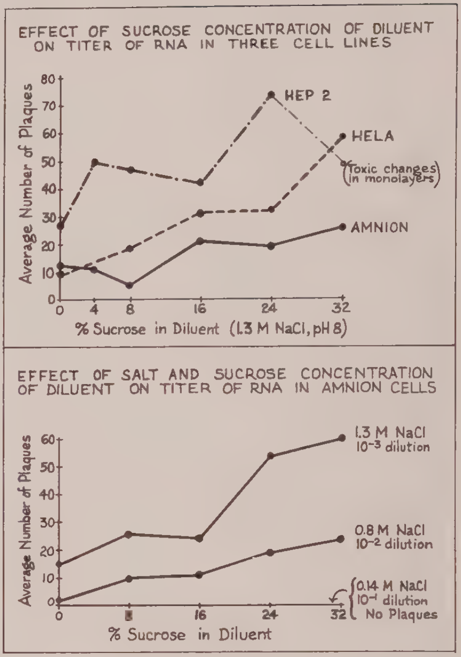

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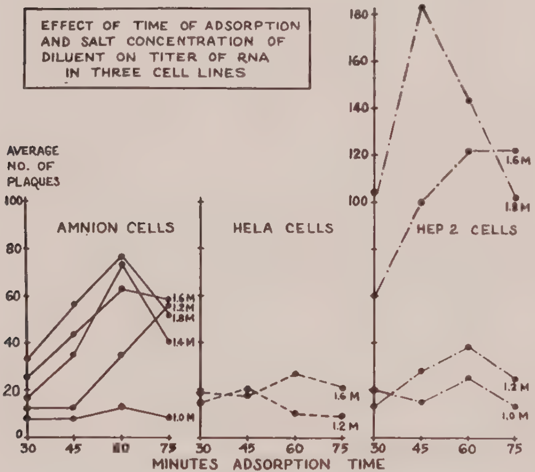

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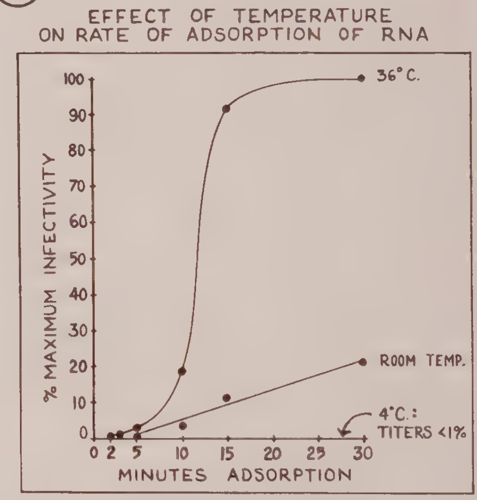

④

FIG. 1-4

The titer of whole virus is not significantly different as measured in the 3 cell lines and is not influenced by the salt concentrations used in the experiments on RNA.

Influence of salt concentration and time of adsorption on infectivity of poliovirus RNA in 3 cell lines. The same effect of cell line was demonstrated in experiments which tested prolongation of adsorption time at varying salt concentrations. In amnion and HEP_2 cells, there was a significant increase in degree of infectivity of RNA with time between 30 and 60 minutes after seeding and with increase in salt concentration above 1 M. In HeLa cells no increase in infectivity occurred with adsorption times longer than 30 minutes, and there was no significant enhancement with salt concentrations above 1 M. These experiments were carried out in the usual fashion except that the cells were washed once with isotonic salt solution containing 10% normal serum (monkey or horse) before being layered with agar. Fig. 2 gives the data from one of 3 such experiments, all of which showed quite similar results. The most notable variable was an increase of RNA infectivity with increase in adsorption time in one of the 3 experiments with 1.2 M diluent in HEP_2 cells and in 2 of the 3 in amnion cells. It is apparent that 1.2 M NaCl is a borderline concentration between the 1.0 M range to which there was uniformly no titer increase with adsorption time longer than 30 minutes and the 1.4 M range in which such titer increase was regularly reproducible.

There is relatively little effect of salt and these increments of adsorption time on titer of whole virus under similar circumstances. This suggests that the differential susceptibilities of the different cell lines to RNA infectivity are more than a measure of the ability of the cell lines to grow poliovirus in the presence of unusual concentrations of salt.

Influence of sucrose concentration on infectivity of polio RNA in 3 cell lines. The effect of sucrose on RNA infectivity was studied to see if there was an additional increase in infectivity due to increasing osmolarity without increasing salt concentration. Again, it was evident that HEP_2 cells showed the greatest sensitivity to altered environment with a definite rise in titer of RNA infectivity with increasing sucrose concentrations up to the point of cell toxicity (usually at 35-45%). The response of HeLa and amnion cells was less regular, but in general HeLa cells seemed a little more sensitive to increasing sucrose concentrations. Increase in RNA infectivity with increase in sucrose seemed relatively greater at higher salt concentrations. This proved to be true in about the degree shown in Fig. 3, in 3 out of 4 experiments. We have not been able to demonstrate infectivity of RNA in isotonic diluent with added sucrose up to 32%. This is in contrast with the findings of Ellem and Colter(8) who found their highest titers with RNA in isotonic salt solution plus added sucrose to 17%.

Influence of preliminary treatment of host cells with solutions of high pH and salt concentrations. To explore the direct effect of salt concentration on the cells in promoting enhanced RNA infectivity, amnion cell monolayers were washed before inoculation with fluids containing varying salt concentrations. The wash fluids were removed as completely as possible before seeding with RNA in either isotonic or hypertonic salt diluents. The results of a typical experiment are shown in Table 1. Preliminary washing with hypertonic saline at pH 8 increased infectivity only if the RNA diluent was suboptimal in molarity and pH.

RNA in isotonic diluent regularly showed a low degree of infectivity following high salt wash however thoroughly the wash fluid was drained from the cell layer (Table 1). Under these circumstances, the only plaques which formed showed such a regular pattern of distribution (either limited to the peripheral gutter of the plate or to a halfmoon area extending from the center of the plate to the gutter) that effect of the residual salt on the RNA molecule rather than on the cell surface could not be ruled out. The localized plaque pattern still formed when the cells were preincubated for 10 or more minutes in a 1.6 M NaCl solution which was thoroughly removed immediately before inoculation. No plaques appeared at all when such pretreated cells were quickly flushed with isotonic solution and inoculated immediately thereafter. Preliminary "conditioning" of the cells with hypertonic salt apparently had no measurable lasting effect on ability of the cells to become infected by RNA in low salt concentrations. Since the "salt effect" on infectivity is known to be very rapid, however, the lack of cell "conditioning" with salt is not decisive in either direction.

Alteration of susceptibility of host cell to RNA by factors other than salt concentration. The influence of factors other than salt con-

TABLE I. Effect of Salt Concentration and pH of Solutions used to wash Amnion Cell Monolayers before Inoculation on Infectivity of RNA Diluted in 0.14 M or 1.3 M NaCl at pH 8.

Salt Concentration of Wash Solution	Number Plaques Produced by RNA Diluted in Salt Concentration Noted and Tested on Monolayers Washed with pH Noted.			
	pH 7.2		pH 8.0	
	.14 M (10^{-2} dil)	1.3 M (10^{-3}/2 dil)	.14 M (10^{-2} dil)	1.3 M (10^{-3}/2 dil)
.14 M	0	32*	0	30
.6 M	.5	28	.7	35
.8 M	2.6	24	3.8	35
1.0 M	14.0	24	16.0	31
1.1 M	14.0	27†	20.0	27
1.3 M	31.0	16†	48.0	30

* Avg number plaques/3 plates. † Toxic changes in monolayer.

centration on degree of infectivity of polio RNA offers additional evidence in favor of the importance of the state of the host cell on degree of infectivity expressed by an RNA.

Effect of age of cells at time of inoculation on susceptibility of the cells to RNA. Within the time limits, 1 to 4 days, age of cells had no effect on cell susceptibility to whole virus. Table 2 shows, however, that monolayers of both amnion and HEP$_2$ cells grown for one day were clearly less sensitive to RNA than cell layers incubated for 2 to 4 days prior to inoculation.

Effect of environment of cells during growth on their susceptibility to RNA. Experiments which used the 2 M $MgSO_4$ diluent and calf serum fed monolayers recommended by Holland *et al.*(9) provided an interesting illustration of the effect of cell environment during growth on susceptibility of the cell to infection by the same RNA diluted in 2 M $MgSO_4$ or in 1.6 M NaCl. These experiments were unusual in that the cells were refed with the feeding solutions indicated in Table 3 twenty-four hours before inoculation, were washed with isotonic solutions after the adsorption period and were layered with agar containing the same serum with which they had been fed originally. Serum concentration was the same throughout except in the "not refed" plates where the final agar overlay contained 10% serum instead of the 20% used in the others. Table 3 presents data averaged from 6 experiments indicating that cell feeding solution had no significant effect when RNA was suspended in NaCl but that there was a marked effect of cell propagating fluid on measure of infectivity of the same RNA suspended in $MgSO_4$. Again, it seems likely that

TABLE II. Effect of Age of Monolayer on Titer of RNA.

Age of Monolayers (Days)	Amnion Cells Exp. No. 1*	2	3	HEP$_2$ Cells Exp. No. 1*	2	3
1	4**	5	7	14	23	25
2	10	31	18	36	62	62
3	21	53	30	24	58	69
4	12	15	26	31	61	88

* Each experiment done simultaneously in the 2 cell lines.

** Avg number of plaques on 4 monolayers.

TABLE III. Effect of Environment of 3 Cell Lines During Growth on Susceptibility to RNA Diluted in 1.6 M NaCl and 2 M $MgSO_4$.

Growth Medium	Plaque Count Produced by RNA Diluted in Following Salt Solutions and Tested in 3 Cell Lines.					
Monolayers refed 24 hrs. before inoc. with Eagle's Soln. + 20%	Amnion Cells		HeLa Cells		HEP$_2$ Cells	
	NaCl	$MgSO_4$	NaCl	$MgSO_4$	NaCl	$MgSO_4$
Calf Serum	12*	23	33	31	24	27
Horse "	18	12	29	22	29	14
Human "	12	13	28	12	24	11
Not Refed	11	4	23	4	26	8

* Plaque count averaged from 6 exp. 3 or 4 plates per lot per exp.

the physiological state of the host cell plays an important role in its susceptibility to infection by RNA.

Effect of temperature on uptake of RNA is considered here because it indicates that the host cells probably play an active role in the process of RNA uptake. Fig. 4 shows the marked effect of temperature on degree of infectivity of RNA following exposure to cells for various intervals at 4°C, room temperature, and 36°. This effect on RNA was somewhat similar to but different in rate and more exaggerated in degree than that on whole virus under the same conditions. Amnion cells were used in the experiment shown. All equipment, the cells, wash solutions and RNA were at the temperature indicated before and throughout the experiment.

Infectivity was maximal in the time interval shown at 36°C, reduced at 25°C, and at 4°C usually less than 1% of that found at 36°. The marked reduction in infectivity at 4°C could be due to diminished molecular mobility and hence diminished chance of contact of RNA molecules with cell surfaces. Against this explanation, however, is the fact that constant machine agitation of monolayers for 45 minutes after seeding did not increase infectivity at 4°C significantly. It seems likely that the process of RNA infection requires metabolic activity on the part of the host cell which is inhibited at low temperatures.

Discussion. Doty and his associates(3), and others before them, showed that the structural configuration of the RNA molecule changes with change in salt concentration, kinking up in high molarities and straightening out and perhaps dissociating in lower molarities and in water. While such alteration in the polio RNA molecule may play a role in its infectivity in tissue culture, the evidence presented here indicates that the effect of hypertonic salt environment on infectivity is at least in part the result of changes in the host cell. In the experiments described in the first part of this paper, the RNA molecule may have changed with the changes in salt concentration, but the influence of salt concentration on degree of infectivity of the same RNA preparation (plaque count) varies in different cell lines.

The data presented in the second part of the paper emphasize that a number of factors in addition to salt concentration may alter cell reaction to RNA to an important degree. It seems likely that such factors as those described and others as yet unknown are playing an important role in producing the variations sometimes found in attempting accurate quantitative work with RNA. Ordinarily, individual monolayers inoculated with RNA measure infectivity as reproducibly as monolayers inoculated with whole virus and relatively little variation in counts is found among plates receiving the same inoculum. From time to time, however, plate to plate variation seems unaccountably large and increasing numbers of plates must be used to achieve overall reproducibility of results. It seems likely that the unexplained variability is due to a variation in the state of the host cell, regardless of all attempts to keep the cell state as constant as possible. To obtain maximal plaque counts, RNA infectivity experiments have to be carried out under conditions visibly damaging to the host cell, and it is logical to assume that the irreversibility of such damage and resultant inability of the cells to support growth of virus will be variable, especially when the cells are not in optimum condition. For this reason, we do not use any of the diluents described here at their maximum concentration tolerated (1.8 M NaCl, 40%$^+$ sucrose, etc.). A diluent of 1.6 to 1.7 M NaCl at pH 8.5 with or without sucrose to 24% gives more reproducible results. Even with all precautions and despite the fact that individual tests with RNA usually look reliable, it is our experience that experiments should be repeated several times to be sure of the dependability of the results. In our hands, at least, measurement of RNA infectivity is not yet as quantitatively reliable as measurement of whole virus infectivity in the same cell system.

It might be said that the experiments described here measure only the ability of the cells to grow poliovirus under the circumstances described. This is not a tenable explanation, however. Whole virus was tested in parallel with most of the foregoing experiments, and the differences described in changing the ability of the cell lines to detect RNA infectivity were not demonstrable for whole virus.

Summary. Evidence presented shows that the influence of salt concentration of the environment on RNA infectivity for mammalian cells is due at least in part to alteration of the state of susceptibility of the host cell. The 3 different cell lines tested simultaneously responded differently when exposed to the same RNA preparation diluted in varying salt concentrations. Other factors which alter host cell susceptibility, sucrose concentration, age of host cell, nutritional environment of host cell and temperature during the adsorption interval, emphasize the importance of the physiological state of the cell at time of exposure to RNA to the degree of infectivity expressed.

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Failure of Reserpine to Cause Depletion of Gastrin in Canine Antral Mucosa.[‡] (27058)

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Although the chemical composition of the hormone gastrin is not known, it is thought to be a protein-like substance of low molecular weight, or possibly a more simple substance attached to a protein molecule. Gastric mucosa is known to contain both histamine(1) and serotonin(2) and these have been considered as possible constituents of gastrin.

Reserpine has the property of stimulating acid secretion from the stomach(3). One possible mechanism for this action is the release of gastrin from the antral mucosa. Reserpine is known to deplete tissue of catechol amines(4), serotonin(5) and histamine(6), and if gastrin were bound in the tissue by the same mechanism as those amines, then reserpine might be expected to release gastrin also.

If the effect of reserpine upon gastric secretion is exerted through release of gastrin, it might be expected that the gastrin content of the antral mucosa would be reduced following its administration. Indirect evidence concerning the storage and binding of gastrin might also be established in this manner.

Methods. Reserpine was administered by mouth in a dose of 5 mg per kg body weight to 13 adult mongrel dogs. Twenty-four hours later the animals were killed and the stomachs immediately removed. Each stomach was opened, washed and mucosa of the antral region dissected from the underlying muscle. The mucosa was stored at −70°C until required.

Extraction. The mucosa was thawed and minced and extraction was performed by the

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method of Gregory and Tracy(7). This involves preliminary treatment with picric acid, followed by extraction with acidified acetone and repeated precipitation and washing with trichloracetic acid. The final product does not contain free histamine. Gastrin was also prepared by this method from the antral mucosa of normal dogs. Linear dose response curves for normal hog gastrin prepared by the Gregory method have been established in this laboratory(8).

Testing. The extracts were tested by subcutaneous injection in conscious dogs with gastric fistula or Heidenhain pouches. The dogs were fasted for 24 hours before the tests. Secretion in response to gastrin normally starts within 30 minutes of the injection and has practically ceased after 3 hours. Accordingly, secretions were collected over a 3-hour period following injection and total output of HCl during this period was determined by titration with 0.1N NaOH using phenol red indicator.

Results. The extract prepared from the reserpinized dogs was divided into 2 portions, each equivalent to 70 g antral mucosa. In the Heidenhain pouch dogs this dose of gastrin caused an output of 2.6 mEq. HCl over a period of 3 hours. The same doses of gastrin prepared from the antral mucosa of normal dogs caused an average output of 2.2 mEq. HCl from the same Heidenhain pouch dogs. In another dog with a fistula from an intact stomach, the gastrin prepared from 70 g of mucosa after reserpine produced a response of 17.8 mEq. HCl over a 3 hour period. It was not possible to establish the response of gastric fistula dogs to gastrin prepared from normal dog mucosa but the response to the gastrin prepared from reserpinized dogs was larger than that obtained from comparable amounts of hog gastrin tested on several occasions.

Discussion. Several methods have been described for preparing gastrin(7,9-15). In all of them a biologically active substance has been obtained from the protein fraction of mucosal extracts, but the products have differed in their chemical properties. Thus, the isoelectric point has ranged from pH 4.5(10) to pH 7(12); gastrin has been described as dialysable(10,14) and non-dialysable(11,12, 13); and as soluble(9) or insoluble(16) in 80% ethanol. These facts suggest that gastrin itself may be a relatively simple substance attached to a protein molecule and that the precise properties of the "gastrin" in mucosal extracts may depend on method of extraction.

Gastrin prepared by any of the methods referred to does not contain significant amounts of free histamine. Recently, Wojciech and Ivy(15) described a new method of preparing gastrin. This gastrin contained no free histamine on biological assay; and the active principle was of large molecular size, or attached to a substance of large molecular size (17). When treated chemically, this gastrin yielded histamine in sufficient amount for a supra-minimal secretory response(18). It was, therefore, suggested that gastrin is histamine or a histamine-like compound bound to another substance. Confirmation of these findings has not been reported.

In the present investigation administration of a large dose of reserpine to dogs did not deplete the antral mucosa of gastrin. Histamine and serotonin are liberated from tissues by reserpine, and the results, therefore, suggest that these substances are not constituents of gastrin, and that the known secretagogue effect of reserpine cannot be attributed to release of gastrin.

Summary. A large dose of reserpine was given to dogs. The dogs were subsequently killed and gastrin was extracted from the antral mucosa. The activity of this gastrin was similar to that of gastrin prepared from normal dogs. This finding is thought to be evidence that histamine and serotonin are not constituents of gastrin, and that reserpine does not stimulate gastric secretion through release of gastrin.

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Studies with Cells From Marine Fish in Tissue Culture.[*] (27059)

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In vitro cultivation of normal and abnormal mammalian and fowl cells has proven a valuable tool for biochemical, physiological and virological studies. The comparatively sparse work on *in vitro* cultivation of cells from fresh water fish is adequately summarized by Wolf and Dunbar(1) as is their recent work with explants of trout and goldfish tissues. More recently both Grutzner(2) and Wolf *et al.*(3) have utilized slight modifications of the technic of dispersion of cells by primary trypsinization for preparation of monolayer cultures of cells from fresh water fish. Wolf *et al.* (4) have used tissue cultures of trout cells for studying the virus causing infectious pancreatic necrosis in the young of that species. To our knowledge, there are no published reports on cultivation of tissues from marine fish. This paper describes the successful *in vitro* cultivation of cells from some marine teleost fish.

Materials and Methods. Tissues from the yellow-striped grunt (*Haemulon flavolineatum*) were used for most of this study due to their abundance in South Florida Waters, although the black angelfish (*Pomacanthus* sp.), porgy (*Calamus* sp.), pork fish (*Anisotremus virginicus*), red snapper (*Lutjanus* sp.), and sand perch (*Diplectrum formosum*) were also used at various times. These fish were all trapped in the Atlantic Ocean or in Biscayne Bay near Miami, Fla. and were kindly furnished for this study by the Miami Seaquarium. All fish were of a size to be considered sexually mature. Fish were killed just before use by severing the spinal column immediately posterior to the head. The tissues to be used were removed with sterile instruments and received three 10 minute washes at room temperature in Hanks' balanced salt solution (Hanks' BSS) containing penicillin (300 units/ml), streptomycin (150 mg/ml), and mycostatin (300 units/ml). Three to 4 explants (1-3 mm^3) per tube were cultured in stationary Bellco pyrex brand culture tubes or Leighton tubes with coverslips, usually on a coagulum of equivalent amounts of 50% chick embryo extract (prepared from 9-day-old chick embryos in Hanks' BSS) and chick plasma (Microbiological Assoc.).

Primary trypsinization was accomplished by mincing the washed tissue into small fragments which were then added to about 10 volumes of 0.25% trypsin (Difco) prepared in Mg^{++} free Hanks' BSS containing penicillin (100 units/ml) and streptomycin (50 mg/ml). In later experiments the trypsin solution contained 0.206M NaCl instead of the usual 0.137M used with mammalian systems. The trypsin was allowed to act on the tissue fragments in an Erlenmeyer flask over a magnamix at room temperature for 30 minutes after which the initial trypsinate was discarded and fresh trypsin added. At 1 and 1½ hours the trypsin plus cells was transferred into human serum (4 volumes of trypsin plus cells and 1 volume of serum) at 5°C and then either (1) separated by centrifugation at 700 rpm for 7 minutes at 5°C and the cells resuspended in growth medium or (2) diluted with an equal volume of growth medium without centrifugation. The cells were then counted in a hemocytometer and seeded at the desired concentration in 1 ml of growth medium per culture tube. Passages were accomplished either (1) by scraping with a rubber policeman into fresh growth medium or (2) by trypsinization according to the following procedure. Four tenths ml of trypsin (0.25%) was added to each tube which was then gently agitated until the cells were off the glass (usually 3-5 minutes). Then 0.1 ml of human serum and 1.5 ml of growth medium were added to each tube. The individual cell suspensions were pooled and the cells counted. The desired cell concentration was then seeded in 1 ml of growth medium per culture tube.

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Growth media used in this study were various modifications of Eagle's Basal Medium with added sera as discussed under results. Each ml of growth medium contained 100 units of penicillin, 50 mg of streptomycin, and 100 units of mycostatin. Calf serum and Eagle's concentrates (100x) were purchased from Microbiological Assoc. Human serum was purchased from Philadelphia Serum Exchange. Grunt serum was obtained by pooling the blood obtained by partial decapitation of 20 fish and separating the serum from the fibrin and cells by centrifugation in the cold. This serum was sterilized by passage through a millipore filter.

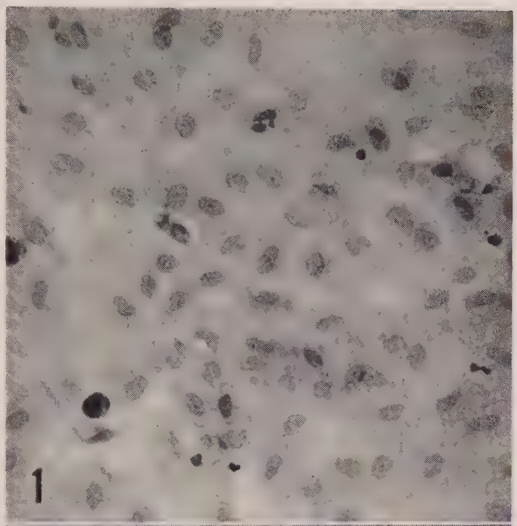

FIG. 1. Epithelium-like cell outgrowth or migration from grunt fin tissue, 3 days after explantation. H+E. 353 X.

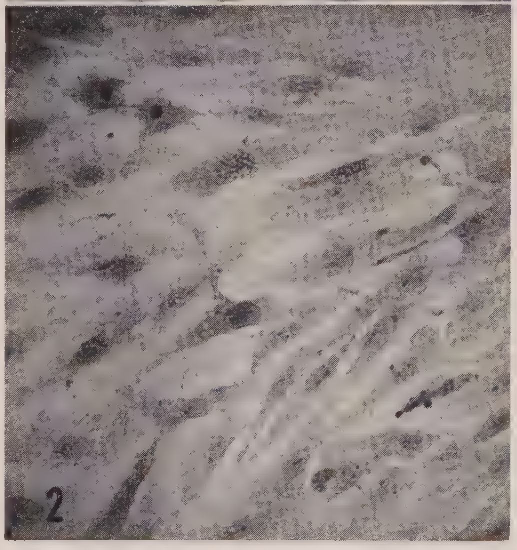

FIG. 2. Fibroblast-like cell outgrowth from grunt fin tissue, 15 days after explantation. H+E. 353 X.

Results. Initial studies with grunt fin explants in Eagle's medium with 10% human serum showed a sequence of outgrowths which in later experiments was also seen when extra sodium chloride was added to the medium. At one day after explantation a sheet of epithelium-like cells appeared around the explant (Fig. 1). This cell type could not be subcultured and began to deteriorate after 5-8 days. A second cell type, fibroblast-like in morphology appeared at 3-5 days and after 3-5 weeks formed an extensive monolayer of cells which could be subcultured. This sequence of outgrowths occurred at 20°C and not at 30°C or 27°C. Media changes at 4-6 day intervals gave apparently the best results. No significant outgrowths occurred when calf serum was used instead of human serum. Fin explant cultures of fibroblast-like cells showed much heterogeneity between tubes but reasonably homogenous sister cultures were obtained upon successful passage (Fig. 2). Some difficulty was encountered in passing these cells and the best adherence was obtained when the recipient tubes were first coated with a thin layer of plasma clot before the cells were added (about $2x10^5$ cell/ml were needed). The importance of a clot surface was evident from findings which revealed that the mere addition of chick serum and embryo extract to the medium without clot formation had no beneficial effect on cells attached directly to the glass. With all successful passages there was an increase in cell number but invariably all attempts at establishment of a cell line ended in death of the cultures, usually after 3-6 passages at 1-2 week intervals.

Explants of fin tissue from the sand perch gave similar results whereas those of the black angel fish fin failed to form monolayers of fibroblast-like cells. The first stage, migration of growth of epithelium-like cells took place, but the fibroblast-like cells which appeared toward the end of this stage did not proliferate and died after 1-2 weeks.

Grunt ovary, heart, and gill cover explants yielded monolayers of fibroblast-like cells which could be passaged. Little or no epithelium-like outgrowth appeared from these tissues. Continuous serial passages have not been undertaken. Grunt swim bladder, skin, liver, spleen, and gill explants failed to yield significant outgrowths.

Initial attempts at preparation by primary trypsinization of grunt fin cells in Eagle's medium with 10% human serum were unsuccessful. Very few cells attached to the glass or plasma clot surface and little if any growth occurred. Since the osmolarity and NaCl content of marine fish sera are higher than those of mammalian sera(5) the possibility became evident that perhaps, in contrast with organized tissue fragments, separated cells dispersed by abrupt exposure to trypsin could not adapt to the mammalian osmotic (ionic) environment as present in Eagle's medium. Typical results obtained when the NaCl content of Eagle's medium with 10% human and 10% calf was increased are shown in Table 1. These results show that the best growth was obtained when the Hanks' BSS used in preparing the growth medium contained about 0.206 molar NaCl (instead of the usual 0.137M used with mammalian cells). Subsequent studies with diluted rather than centrifuged primary cell suspensions indicated that optimal NaCl concentration was about 0.219M. Such cells gave complete monolayers of fibroblast-like cells which could be passaged at 9-10 days. These cells were morphologically indistinguishable from the fibroblast-like cells obtained from explants. In attempting further to define the requirements for culturing primary cells from a marine fish (grunt) the following observations were made. Eagle's basal medium prepared from Hanks' BSS containing 0.206M NaCl and supplemented with 10 or 20% human serum supported the growth of primary fin cells at a somewhat slower rate than did a medium of similar composition but containing a mixture of 10% human and 10% calf serum. Ten or 20% calf serum when used as the sole supplement was unsatisfactory although this medium appeaerd to be an adequate maintenance medium even without the added NaCl. The use of grunt serum with or without human or calf serum proved to be unsatisfactory for growth of primary grunt fin cells. With primary cell suspensions prepared without the use of centrifugation adequate growth could be obtained with $4\text{-}5x10^5$ cells/ml but with centrifuged suspensions growth was not obtained with seeds of less than $8x10^5$ cells/ml. Media changes at 4, 8, and 12 days yielded satisfactory results. Of the various types of culture surfaces used with primary trypsinized cells, pyrex brand culture tubes (Bellco) and plastic tissue culture bottles (Falcon) appeared most satisfactory. Soft glass prescription bottles and flint glass tubes would allow attachment and growth but the fibroblast-like cells tended to

TABLE I. Growth of primary grunt fin cells* in Eagle's Basal Medium supplemented with 10% human serum, 10% calf serum and at various concentrations of sodium chloride.

Molar. conc. NaCl†	Condition of cultures after 13-16 days at 20°C.
.137	Few cells attached to glass surface—not proliferating.
.206	Complete monolayers of fibroblast-like cells can be successfully passaged.
.264	Incomplete monolayers of fibroblast-like cells—can be passaged later.

* These cells were separated from the trypsin by centrifugation and were seeded at 8 x 10^5 cells/ml.

† Concentration of NaCl in Hanks' Balanced Salt Solution used in preparing media.

fall back creating large holes in the cell sheet. The required temperature for growth of primary trypsinized cells was in the range of 20-22.5°C. Maximal cell yields (up to 1.5×10^6 cells/tube) could be obtained after 3-4 weeks but most passages of primary cells were accomplished earlier (10-14 days) due to the better quality of the cells at this time. Such monolayer cultures can be serially passaged at 7-10 day intervals with 2-5 fold increases in cell number. One such line has been passaged 24 times over the past 9 months. Several passages of this line have been accomplished with seed levels as low as 25,000 cells/ml although the usual seed levels were around 10^5 cells/ml. The use of Eagle's Basal Medium supplemented with a mixture of human and calf sera with additional NaCl has proved successful for cultivation of trypsin dispersed fin cells of the porgy and the pork fish but not of the red snapper.

Discussion. The results reported here as the initial findings of a continuing study on marine life are apparently compatible in many ways with those obtained by Wolf *et al.*(1,3) for terrestrial and aquatic poikilothermic vertebrates. The basic growth sequence from explants of fin tissue of marine fish was very similar to that reported for fresh-water fish. This consisted of an initial outgrowth (or migration) of epithelium-like cells which could not be subcultured followed by appearance of a fibroblast-like cell type which was capable of being subcultured with a subsequent increase in cell number. The requirement for incubation temperatures of about 20°C and the ability to grow in media designed for mammalian cells were other points of similarity between marine and non-marine vertebrates. The important difference between the tissue culture conditions essential for cell from these 2 types of animals was the requirement for an increased sodium chloride content of the growth media when initiating primary cultures of trypsin dispersed cells from marine fish. The additional NaCl was also necessary in order to serially subculture marine fish cells; if mammalian levels of NaCl were used, the marine fish cells died after a few passages regardless of the method used to initiate them. Wolf (personal communication) has successfully subcultured fresh water trout gonad cells for over 20 passages using mammalian media. Studies are currently in progress to determine more exactly the total ionic requirements for culturing cells from marine fish, although it appears that the levels of other ions (Ca^{++}, Mg^{++}, K^{+}) in mammalian media are adequate to support sustained proliferation of such cells.

Since our primary objective was to develop methods for culturing cells from marine fish to be used for isolation of marine viruses, it was necessary to be able to initiate large numbers of duplicate cultures. Successful cultivation of trypsin dispersed primary cells and establishment of a serially subcultured line of cells from the grunt has facilitated production of replicate monolayers of cells not only for virus studies but also for physiological and biochemical studies. One point of interest in latter respect is the large numbers of trypsin dispersed cells required (4×10^5) to initiate primary cultures as compared with the much smaller number (2.5×10^4) which sufficed for successful subcultures. The reasons for this difference are not known but could possibly be explained on the basis of (a) the majority of the cells in the initial primary preparation are unable to divide and grow, (b) consequent selection of cells capable of *in vitro* growth under the conditions used, and (c) possible adaptation of the cells to this *in vitro* environment. On the basis of routine microscopic observation it can be stated that the initial attachment to the glass or plastic surface was poor, thereby accounting in part for some of the delay in complete monolayer formation with primary cells. The need for further studies on the nutritional aspects of marine fish cells is evidenced by the apparent species differences regarding *in vitro* growth.

Summary. Cells of tissues obtained from marine fish were shown to grow by the explant method at 20°C in a medium designed for mammalian cells. Preparation of monolayers from cells dispersed by primary trypsinization was accomplished when additional sodium chloride was added to mammalian cell media. Cells in continuous culture were found to multiply more efficiently (as regards time required for sheet formation and number of cells required for satisfactory growth) than did cells in primary culture.

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Action of Ammonium Ion on an Early Phase of Viral Infection.* (27060)

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In tumor cells influenza virus produces high titers of hemagglutinins but little infectious virus(1). This indicates an incomplete replication cycle with no spread of virus among cells. A previous report has shown that Krebs 2 ascites cells liberate ammonia from glutamine, and the antiviral effect of immediate or delayed treatment with these substances was described(2). The purpose of the investigations reported here was to obtain information about the nature of the initial process of infection during which there is a short period when viral growth may be inhibited by addition of ammonium ions to the medium. Later treatment is without effect.

Method. The medium, the method of preparing the ascites cell suspensions, and determination of cell-associated hemagglutinins have been described(2). To suspensions containing 3.2×10^6 tumor cells per ml the PR_8 strain of virus was added at a concentration of 16 to 32 hemagglutinating units (HA) per ml, giving an input multiplicity of the order of 10 E.I.D.50 per cell. Cells were allowed to adsorb virus for definite periods of time, then washed and changed to fresh medium. If a single cycle of viral replication is assumed, the HA yield reflects the proportion of cells originally infected. As judged by HA titers at 24 hours about 15% of susceptible cells were infected within 10 minutes, and virtually 100% between 2 and 4 hours. Reducing the temperature of incubation from 35°C to 4°C caused only a slight diminution in rate of viral attachment to cells. Maximum HA titers obtainable in this system were 1,000 to 2,000 units per 0.1 g of extracted cells.

Ammonium phosphate was added at a concentration of 0.01% and unless otherwise noted remained in the medium during the subsequent incubation period. Disappearance of NH_3 nitrogen from the fluid indicated an uptake by cells during the first 4 hours of about 100 μg per gram wet weight.

Results. To determine the length of the period of sensitivity to NH_4^+, cells and virus were kept at 4°C for 2 hours, then the cells were washed and suspended in medium warmed to 35°C. In the tubes receiving ammonium phosphate at 10 minutes the 24-hour HA titers were 32, in those treated at 15 minutes 128, and at 40 minutes 256 (control titer 512). This experiment shows quite clearly that at 35°C the period after adsorption during which viral growth may be significantly inhibited by NH_4^+ is only a few minutes.

When cells are kept with virus at 5°C or 15°C for a period as long as 4 hours then washed and treated immediately with NH_4^+, marked inhibition of HA formation is obtained (Table I). This indicates that low temperature prolongs the period of NH_4^+ sensitivity in contrast to its slight effect on attachment of virus. At both 25 and 35°C, however, the period of highest NH_4^+ sensitivity is about 10 to 20 minutes.

Since low temperature delays the process by which virus becomes refractory to NH_4^+, active metabolism of the host cell is probably a necessary factor in this reaction. Omitting the substrate during preincubation did not

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TABLE I. Period of ammonium sensitivity as affected by temperature, medium, and inhibitors.

Preincubation with virus Time hr	Temp. °C	Medium*	HA titer after 24 hr with NH_4^+ as % of control
4	5	gl + gc	0.4
4	15	“ “	1.5
¼	25	“ “	3
1	25	“ “	100
¼	35	“ “	$<$ 0.4
¾	“	“ “	100
1	“	BSS only	50
2	“	gl + CN 60	$<$ 3
2	“	gm + DNP	$<$ 6
1	“	gl + CN 30	3
4	“	“ “ “	25
1	“	gl + gc + CN 60	100
1	“	“ “ + DNP	100

* Hanks' balanced salt solution (BSS) with substrates and inhibitors as follows: gl, glutamate 1 mg/ml; gc, glucose 1 mg/ml (with bicarbonate at 0.14%); gm, glutamine 1 mg/ml; CN, potassium cyanide (pH 7.6) 30 or 60 μg/ml; DNP, 2-4 dinitrophenol 50 μg/ml.
$(NH_4)_2HPO_4$ at 0.01% in all experiments.

appreciably increase the period of NH_4^+ sensitivity, probably because the cells contain a reserve of metabolites. Consequently studies were done with cyanide and 2-4 dinitrophenol in media with and without glucose to determine whether oxidative or glycolytic sources of energy were essential. Cells were incubated with virus in the presence of these inhibitors for short periods then washed and suspended in the glutamate-glucose medium. To one set of tubes containing cells which had been infected in the presence of cyanide or DNP ammonium phosphate was added. The other sets containing treated infected cells received no NH_4^+ and served as controls for the effect of the inhibitors by themselves. In these the HA yields reached 128 to 256, indicating only a slight reduction in titer from CN or DNP alone. In the cell virus system first treated in absence of glucose with cyanide at 60 μg/ml or DNP at 50μg/ml for 2 hours then placed in a medium containing NH_4^+ no hemagglutinins were formed (Table I). Cyanide at 30 μg/ml under similar conditions also increased the period of NH_4^+ sensitivity. Interference with oxidative metabolism of the cells, like low temperature, has the effect of prolonging the period of NH_4^+ sensitivity. When, however, the preincubation medium contained glucose with cyanide or DNP, the period of NH_4^+ sensitivity was less than an hour (last lines of Table I).

To test for reversibility of the effect of NH_4^+, cells, virus, and ammonium phosphate were incubated together for varying periods of time from 1 to 18 hours. The cells were then washed and suspended in fresh medium without virus or NH_4^+ for a further incubation period of 24 to 48 hours. As the results presented in Table II show, even after an initial period of a few hours' exposure to NH_4^+ there is interference with subsequent HA formation, and at 18 hours most of the virus capable of replication seems to have been lost. As shown previously, cells incubated by themselves with NH_4^+ for 24 hours retain the ability of supporting some viral growth and ammonium phosphate at a concentration of 0.01% does not reduce the infective property of extracellular virus(2).

TABLE II. Reversibility of effect of ammonium ion and action of antibody.

Period of exposure to NH_4^+. (hr)*	HA titer** a. NH_4^+.	b. Control	a/b × 100
1	128	256	50
2	256	1024	25
4	128	2048	6
18	4	1024	1.5
4 (ab 50)	$<$4	32	—
4 (ab 10)	32	512	—

* Virus added at 0 hr.
** 24 to 48 hr after removal from medium with NH_4^+. Ab 50, ab 10 indicate that NH_4^+-treated infected cells and controls were placed in fresh medium containing 50 or 10 HA-inhibiting units of antibody.

The poorly defined mechanism by which animal viruses reach the interior of cells may involve ingestion (pinocytosis) or enzymatic activity. To ascertain if the presence of NH_4^+ caused attached virus to remain on the surface and hence accessible to antibody, cells were incubated for 4 hours with virus and NH_4^+, then placed in medium containing 10 to 50 HA-inhibiting units of antibody.† Reduction by antibody of cell-associated HA after 24 hours' incubation appeared to be similar in degree in NH_4^+-treated cells and controls (compare lines 3, 5, and 6, Table II) indicating that NH_4^+ did not increase the proportion of neutralizable virus. This procedure has been used to demonstrate delayed penetration of virus in other studies(3). Also receptor-destroying enzyme has been used by Wagner(4) to show the temperature dependence of the penetration phase.

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† Before extraction for titration of cell-associated hemagglutinins cells were routinely washed twice(1).

With influenza in minced chorioallantoic membrane a similar early suppression may be demonstrated by using higher concentrations of NH_4^+; but the increased NH_4^+, even when added after 4 hours, reduces HA yields to about 25% of the control. This is attributed to a toxic effect during the subsequent 24-hour incubation period.

Discussion. Jensen, Force and Unger(5) describe with influenza virus in another species of cell results essentially similar to ours(2). Using a stable line of epithelial cells originating from dog kidney, they were able to demonstrate protection by ammonium ions against the cytopathic effect of the PR8 strain. Similarly glutamine from which ammonia is formed will in the presence of bicarbonate protect Krebs 2 cells against the oncolytic action of influenza virus(2,6). These observations suggest that NH_4^+ at appropriate concentration may have a chemoprophylactic effect in certain cell-virus systems maintained *in vitro*. The concentration of NH_4^+ of 0.005% to 0.01% found to be non-toxic to cells in tissue culture but inhibitory for virus(5) is, however, greatly in excess of tolerated blood levels in the intact animal.

The ammonia effect seems to be most easily demonstrable under conditions favoring formation of large amounts of incomplete virus, although reduction of infectivity titers has been reported(5). Preliminary results with cultures of chorioallantoic membrane and influenza virus suggest that suppression of viral growth by NH_4^+ or glutamine may also occur in cells which produce complete infectious virus.

The process affected by NH_4^+ seems to require cellular energy production, but when oxidative metabolism is inhibited the requisite energy appears to be furnished by glycolysis. Because of the short duration of the period of NH_4^+ sensitivity after adsorption of virus, consideration can be given only to a mechanism which involves initial integration of the viral inoculum with cytoplasmic or nuclear components of the host cell. Ammonia may modify a biosynthetic process essential to viral invasion.

Summary. Following adsorption of influenza virus by Krebs 2 ascites cells there is a brief period of sensitivity to inhibition of viral replication by ammonia. After an hour at 35°C addition of ammonium phosphate causes no inhibition. The period of ammonium sensitivity is prolonged by low temperature, cyanide, or 2-4 dinitrophenol, but the presence of glucose in the medium prevents this effect of CN and DNP. The results suggest that the ammonium-sensitive phase of infection is an energy-requiring process which occurs soon after adsorption and penetration, and comprises initial integration of the viral inoculum with cell components.

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Transfer of Allergic Encephalomyelitis Using Splenectomized Albino Rats.** (27061)

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Allergic encephalomyelitis (AE), induced in rats and other animals by injection of spinal cord plus Freund's adjuvant, has been under study in our laboratory as a model for

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* Recipient of Investigatorship of Health Research Council of City of New York.

defining immune mechanisms implicated in autoimmune disease(1). Although AE is believed on good grounds to have an immunological mechanism, the nature of the immune agent directly responsible for the characteristic encephalomyelopathy has remained unknown. The transfer of AE from spinal cord-sensitized donors to normal animals with cells or serum provides one means of clarifying whether the responsible immune agent is cellular or humoral in nature. Chase(2) has reviewed the past unsuccessful efforts of various investigations to achieve transfer of AE with serum or cells and using intact, randomly bred animals. Our attempts to accomplish transfer in this fashion have given similarly negative results(3).

Lipton and Freund(4) reported that AE may be transferred in rats by means of parabiosis. Paterson(5) subsequently described successful transfers of AE in randomly bred albino rats using lymph node cells (LNC) of sensitized donors and recipients which had been rendered immunologically tolerant by pretreatment with normal rat spleen cells. Best results were noted in recipients that received normal spleen cells of splenectomized prospective LNC donors. Our success was attributed, in part, to the use of tolerant recipients in which the donor LNC, by not being rejected by the recipient, could survive and function for a longer period of time and, thus have a better opportunity to react with the nervous tissue of the recipient.

Continued search for other methods of AE transfer in this laboratory have included: injection of donor LNC either into the carotid artery or into the brain of recipients, prolonged cross-circulation of donor-recipient pairs, use of heavily X-irradiated recipients and highly inbred intact rats. These methods of transfer gave only an occasional convincing positive transfer.

Our previous success in transferring AE with LNC from splenectomized donors(5) suggested to us that the spleen might well play a role in AE and its transfer. Initial experiments indicated that, indeed, splenectomy of sensitized donors permitted LNC transfer of AE to intact albino recipients. While our work was in progress, Koprowski et al(6) stated that splenectomy facilitated LNC transfer of AE within a highly inbred strain of rat. Their report did not make clear whether splenectomy of both donor and recipient gave better results than splenectomy of only the donor or only the recipient.

The present report is an extension of our earlier study(5) and describes the transfer of AE using LNC and splenectomized, commercially available albino rats. Our data agree with those obtained by Koprowski et al(6) in an inbred strain of rat and, in addition, indicate that splenectomy of only the donor is sufficient to facilitate transfer of AE to intact recipients.

Materials and Methods. The animals employed as donors and recipients were CFN ("specific pathogen free") Wistar rats† weighing between 125-175 g each. All techniques concerning splenectomy, intracutaneous sensitization of donors with guinea pig spinal cord plus adjuvant, preparation of donor LNC suspensions and processing of recipient brains and spinal cords for microscopic examination were carried out as previously described(5). Recipients routinely received 1 to 2 mg of heparin†† intravenously a few minutes prior to intravenous injection of donor LNC suspensions to prevent thromboplastin death. LNC preparations were periodically stained with methylene blue dye to assess their viability, and approximately 80% of the cells in a given preparation appeared viable as judged by their failure to take up this dye. Ratios of donor to recipients were usually 1:1; in one transfer experiment this ratio was 1:2.

Results. The results of 3 transfer experiments are shown in Table 1. Transfer of LNC from 11 splenectomized donors sensitized to spinal cord plus adjuvant to 12 splenectomized recipients gave positive results in 5 of these recipients. Each of these 5 recipients was found to have a few to numerous focal areas of vascular and perivascular inflammation characteristic of AE readily demonstrable with the brain or/and spinal cord. Transfer of LNC from 6 splenectomized donors to 6 intact (non-splenectomized) recipients also gave

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† CFN albino rats were obtained from Carworth Farms, New City, N. Y.

†† Heparin Sodium, 1000 units (app. 10 mg) per ml, Connaught Medical Research Laboratories, Toronto, Canada.

TABLE I. Transfer of Allergic Encephalomyelitis (AE) in Splenectomized Rats using Lymph Node Cells.

Donor Rats*		Recipient Rats†		
No. of Rats	Splenectomized	No. of Rats	Splenectomized	AE Lesions
11	+	12	+	5/12‡
6	+	6	0	2/6
6	0	6	+	0/6
5	0	5	0	0/5

* Donor rats splenectomized 7-14 days before sensitized to guinea pig spinal cord plus Freund's adjuvant; lymph node cells obtained 6 or 7 days after sensitization.

† Recipient rats splenectomized 7-12 days before injection of donor lymph node cells; sacrificed for histological examination 10 or 11 days after single intravenous injection of from 200 to 500 x 10^6 donor lymph node cells.

‡ Numerator—No. of recipients with AE lesions; denominator—No. of recipients used.

clearcut positive results in 2 of these 6 recipients. In contrast, transfer of comparable numbers of LNC from 6 intact donors to 6 splenectomized recipients gave negative results, none of the 6 recipients having demonstrable microscopic abnormalities within their central nervous system. Transfer of comparable LNC from 5 intact donors to 5 intact recipients also gave negative results in agreement with our past attempts to transfer AE in intact albino rats. The data (Table 1) concerning transfer of AE from splenectomized donors to intact recipients have been fully confirmed, viz., of 6 additional intact recipients which received LNC of 6 splenectomized and sensitized donors, respectively, 2 of the 6 recipients were found to have characteristic lesions of AE.

None of the recipients exhibited clinical neurological signs of AE following LNC transfer, including the 7 animals which had typical AE lesions. This observation was consistent with the uncommon appearance of paralysis noted previously in tolerant albino rats(5). It may also be noteworthy that the success of transfer in these experiments (Table 1) as judged by the proportion of recipients having demonstrable lesions of AE, was less than that previously noted using pretreated and presumably tolerant albino recipient rats.

Control LNC transfers using splenectomized donors sensitized to guinea pig kidney plus adjuvant or adjuvant only and transfer of killed LNC (heated at 56°C × 30 min) obtained from splenectomized donors sensitized to spinal cord plus adjuvant have given negative results.

Discussion. The data (Table 1) indicate that AE may be transferred in splenectomized albino rats using LNC of donors sensitized to spinal cord plus adjuvant. The disease may be transferred with LNC of splenectomized donors to either splenectomized or intact (non-splenectomized) recipients. In contrast, LNC of sensitized intact donors do not appear capable of transferring AE to splenectomized or intact recipients. These results suggest that splenectomy of donors plays a more important role in facilitating AE transfer than does splenectomy of recipients.

Preliminary work using an inbred strain of rat (Fisher 44)††† confirms in full the observation of Koprowski et al(6) that splenectomy facilitates AE transfer in inbred rats. Our work to date with the Fisher 44 strain suggests that it is the splenectomy of the donor that facilitates transfer, since splenectomy of the recipient alone exerts little, if any, influence on the outcome of LNC transfer. In contrast to our transfers employing albino rats, we have noted appearance of hindleg paralysis and particularly intense disseminated lesions of AE in Fisher 44 rats following injection of LNC from splenectomized Fisher 44 donors. This observation provides further support for the thesis previously stated(5) concerning the importance of histocompatibility between donor and recipient with respect to success of AE transfer with LNC.

The role of the spleen in AE and its transfer is under study. It is conceivable that the spleen acts as a "trap" for circulating LNC and that by removing the spleen a greater number of LNC potentially capable of transferring AE are permitted to circulate and return to lymph nodes from which they originated. If such were the case, lymph nodes of splenectomized and spinal cord-sensitized donors might well contain a larger population of "sensitized" cells capable of transferring AE than found in lymph nodes of non-splenectomized donors. It is also possible that the spleen may produce the factor (presumably antibrain antibody) present in immune sera of rats recovered from

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††† Fisher 44 strain of rat, known to be highly inbred, provided by Nat. Inst. Allergy and Infect. Dis., N.I.H., Bethesda, Md.

AE which Paterson et al(7) found effective in suppressing or preventing AE when passively administered to actively sensitized rats. Splenectomy might result in less production of this circulating factor and thereby permit LNC of a sensitized donor to be more efficacious in transfer.

It should be reemphasized here that the present, as well as our earlier(5), transfer studies in rats do not settle the question as to whether "sensitized" cells (delayed or tuberculin-type hypersensitivity) or conventional antibodies are directly responsible for initiating the damage recognized as AE. For it cannot be said as yet whether the lesions in recipients result from antibodies elaborated and shed by donor lymph node cells after their transfer or by interaction of the donor cells directly with antigen in the recipient's own nervous system. The simplified method of transferring AE in commercially available rats, described here, may help in answering this question.

Summary. Previously described methods of transferring allergic encephalomyelitis in commercially available randomly bred, albino rats sensitized to nervous tissue plus adjuvant, entailed use of parabiosis or pretreatment of recipients with normal spleen cells of prospective sensitized lymph node cell donors. Further study indicates that the disease may be transferred using lymph node cells of splenectomized donors sensitized to nervous tissue and either intact or splenectomized recipients. Splenectomy of only the recipient does not appear to facilitate transfer of the disease.

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Mann-Williamson Rat. (27062)

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The Mann-Williamson (M.W.) operation in the dog has been widely used in the study of experimental ulcer(1). We have succeeded in producing ulcer in the rat by a modified M.W. operation; the method and subsequent observations are reported here. We have used rats because they are cheaper than dogs and large numbers can be kept in relatively small space; also, the fact that anatomy and physiology of the stomach of the rat differ from that of the dog made the study desirable.

Methods. Fig. 1. Albino rats of Sprague-Dawley strain (both sexes) with initial weights between 172 g and 512 g (average 284 g) are fasted for 24 hours, water being given *ad lib.* The animal is etherized, and under clean but not aseptic precautions the abdomen is opened by a small incision in the upper midline; the pylorus is ligated and cut just proximal to the ligature. A 5-0 black silk thread is passed through the upper edge of the gastric stoma and secured with a small hemostat. The duodenum is pulled up; the bile duct with many pancreatic ducts entering it is seen coursing through the mesentery; it opens into the duodenum 2 to 3 cm below the pylorus. The jejunum is transected about 1

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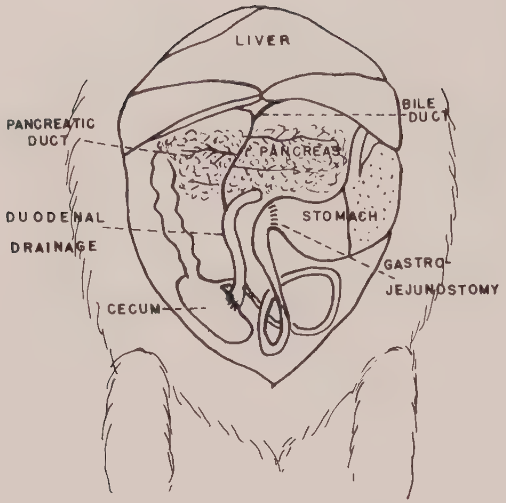


FIG. 1. Diagram of Mann-Williamson operation in the rat.

cm below the ligament of Treitz, and its caudal end is anastomosed end-to-end to the stomach with continuous suture, using the thread previously passed through the stomach; 10 stitches are adequate, serosal suture is not necessary. Next, the apex of the cecum is pulled up and incised 5 mm. The proximal end of the cut jejunum is anastomosed end-to-end to the incision in the cecum with continuous suture. No clamps are used, and usually little or no bleeding occurs. Five ml of 0.9% saline is injected into the peritoneal cavity before closure, and after operation Combiotic is given s.c.

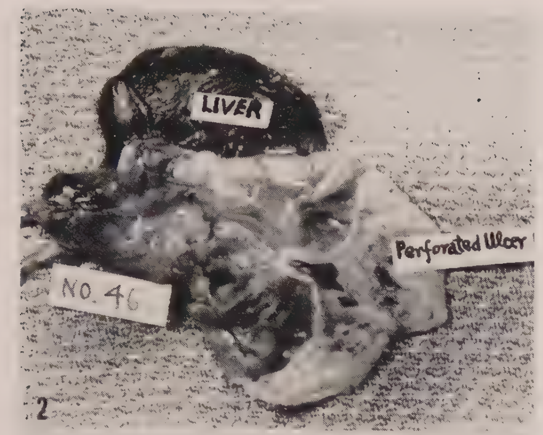
Results. Table 1. Results are divided into 2 groups; A, animals that died or that were moribund and were killed after operation (4 to 11 days, average 7 days), and B, animals that survived longer (13 to 160 days, average 68 days). The reason for this division was the fact that in animals of group A most ulcers occurred in the prostomach, in group B most ulcers occurred at the gastrojejunal stoma and none were found in prostomach. Rats in Group A lost 33% body weight, an average of 13 g per day, and rats in Group B lost 48% body weight, an average 3.3 g per day. The incidence of ulcer was approximately equal in both groups (88% and 85% respectively). In Group A, 67% of ulcers were in prostomach, while in Group B, no ulcers were in prostomach and 87% were at the stoma. Most ulcers in glandular stomach, stoma, and jejunum were single, but in the case of the prostomach, most ulcers were multiple. Most ulcers were round, few were oval, and their size varied from 1 to 10 mm in length or diameter. The largest ulcers in both groups were found at the gastrojejunal stoma. Small erosions and punctuate hemorrhages, mainly in the glandular stomach, were seen in both groups. A number of ulcers in both groups had perforated. Fig. 2 shows multiple ulcers in prostomach of an animal of Group A, Fig. 3 shows a penetrating marginal ulcer in Group B, Fig. 4 shows section of deep marginal ulcer in Group B.

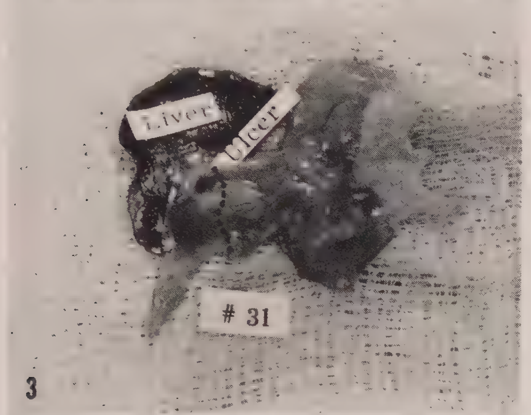
Discussion. The percentage of M.W. ulcers in the rat is approximately the same as in the dog(1). We have sought an explanation for the different distribution of ulcers in Groups A and B namely, the prevalence of ulcers in the prostomach in the early death Group A and of ulcers at the stoma in the late death Group B. The rats in Group A lost approximately 13 g of body weight per day within a short period of time, while the rats in Group B lost only 3.3 g per day over a longer period of time. In Group A, obstruction at the gastrojejunostomy was noted in some of the animals, apparently due to edema and fibrous contraction at the suture line. It is possible that edema may have been present in more animals of that group, and that it disappeared by time of autopsy. If this were the case, increased intragastric pressure and gastric distention would have contributed to formation of ulcers in the forestomach, as we have de-

TABLE I. Occurrence of Ulcers.

Group	No. of Rats	Avg Survival Days	No. of Rats with No ulcer	No. of Rats with Ulcer	No. of ulcers Prostomach	No. of ulcers Gland.Stom.	No. of ulcers Stoma	No. of ulcers Jejun.
A	17	7	2	15	10	3	5*	0
B	27	68	4	23	0	1†	20	8‡

* also 3 ulcers in prostomach. † also ulcer in stomach and jejunum. ‡ also 5 ulcers in stoma.





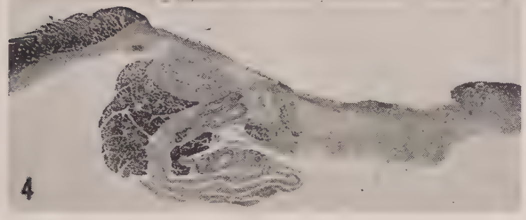


FIG. 2. Early death group (A). Stomach of rat with multiple ulcers in prostomach, one of them perforated. Large ulcer at cardia. Died 7 days after operation.

FIG. 3. Late death group (B). Stomach of rat with one large ulcer at suture line, penetrating into liver. Suture line indicated by dots. Died 107 days after operation.

FIG. 4. Late death group (B). Section through ulcer of stomach of rat with 1 large ulcer at suture line. Floor of ulcer contains dense fibrous tissue with eosinophilic stain. Died 66 days after operation.

scribed in the pylorus ligated mouse(2).

In Group B, most ulcers appeared at the stoma. We are inclined to believe that these ulcers are caused by a deficient blood supply to the region of the anastomosis. Berg(3) has pointed out the relative paucity of arteries in the stomach of the rat, and the lack of adequate collaterals. Injected specimen of rats' stomachs supports our assumption that marginal ulcer in the M. W. rat may well be due to circulatory deficiency(4). Thus it may be possible that marginal (anastomotic) ulcer is caused as much or more by inadequate blood supply than by acid-pepsin, an idea which we have suggested earlier(2,5-7). In both groups, but particularly in Group A, starvation, loss of weight, and malnutrition, may have contributed to the production of peptic ulcer. Anastomotic ulcers were larger and appeared to be older than jejunal ulcers.

We believe that the results of Group B, the animals with prolonged survival time and with a high incidence of ulcers at stoma and jejunum, represent the typical pathology of the M.W. rat.

Summary. The Mann-Williamson operation has been applied to the rat; gastric, marginal, and jejunal ulcers were seen in 85% of the animals. Early postoperative deaths may have been due to obstruction at the gastro-jejunal anastomosis; these animals developed a high incidence of ulcers in the prostomach. In rats that lived longer, the majority of ulcers were found at or nearby the gastro-jejunal anastomosis. It is felt that this latter group is representative of typical Mann-Williamson ulcer in the rat. A possible role of vascular factors in the production of ulcer is discussed.

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Serotonin Release by Bacterial Endotoxin.* (27063)

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Indirect observations have implicated serotonin in shock due to bacterial endotoxin(1,2). Armin and Grant observed that *E. Coli* endotoxin injection produced constriction in the vessels of the rabbit ear which resembled that produced by serotonin(3). During the constriction phase they were able to find minute quantities (0.01 to 0.02 μg/ml) of an ultrafilterable substance in plasma which resembled serotonin in action on the cat uterus, including antagonism by L.S.D. Their findings were later confirmed by others(4) who found 0.004 to 0.01 μg/ml of plasma serotonin 1-2 hours after endotoxin. In addition, many studies had clearly shown that serotonin was released by antigen from platelets of sensitized rabbits (5, 6) and was implicated in anaphylaxis in certain species(7). Previous studies in our laboratories using dogs showed an immediate fall in serum serotonin concentrations, but only small, and not statistically significant elevations in platelet free plasma serotonin(8). The present studies were done to see if immediate release could be demonstrated in rabbits, and if a dose-response relationship existed *in vivo.*

Methods. The carotid artery and external jugular vein of anesthetized (nembutal 30 mg/kg) 3-4 kg New Zealand white rabbits were isolated and cannulated with #240 Clay Adams polyethylene cannulae. Purified *E. coli* endotoxin(9)† was given by intravenous injection. Arterial samples were collected in siliconed tubes containing 50 U.S.P. units of heparin sodium per 6 ml blood for platelet counts and plasma serotonin concentrations. Immediately after collection, blood for assay of plasma serotonin was centrifuged at 4°C for 30 min at 3000 r.p.m. Serum serotonin was collected by the method of Davis(10) and all serotonin samples were assayed fluorometrically(11) using the Aminco Bowman Spectrophotofluorometer. Platelet counts were done in duplicate using Rees-Ecker diluting fluid and a direct counting technique.

Results. Rapid intravenous injection of *E. coli* endotoxin caused thrombocytopenia at all doses employed (Table I). The fall in platelet count was more precipitous with larger doses 15 seconds after endotoxin, but after 5 minutes little difference in counts was present, except at the lowest and highest dose employed. The decrease in total serotonin, measured in serum, also was greater with larger amounts one minute after endotoxin; after 5 minutes the decrease was essentially equal at all doses used (Table II). Plasma serotonin (platelet free) rose to a maximum level 15 seconds after endotoxin, then fell during the first 5 minutes (Table III). No elevation of plasma serotonin occurred with the lowest dose employed (0.01 mg/kg). Separate studies showed that intravenous injection

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TABLE I. Mean Carotid Artery Platelet Counts Following Intravenous Injection of *E. coli* Endotoxin.

Time after Endotoxin		Dose of Endotoxin (mg./Kg.)				
	Saline Control (5)	.01 (5)	.05 (5)	.25 (5)	1.0 (10)	4.0 (10)
Control	326,000	390,000	377,000	291,000	513,000	497,800
15 sec	375,000	485,000	326,000**	207,000**	340,000*	Clumps
60 "	356,000	305,000**	117,000*	30,000*	41,000*	45,600*
5 min	352,000	158,000*	78,000*	38,000*	69,000*	191,000*

No. of animals indicated in parentheses.
* Magnitude of decrease significantly greater than saline control at each indicated time ($p<.05$).
** Magnitude of decrease not significantly greater than saline control at each indicated time ($p>.05$).

TABLE II. Mean Serum Serotonin Concentrations (μg/ml) in Carotid Artery Blood Following Intravenous Injection of *E. coli* Endotoxin.

Time after Endotoxin		Dose of Endotoxin (mg./Kg.)				
	Saline Control (5)	.01 (5)	.05 (5)	.25 (5)	1.0 (10)	4.0 (10)
Control	6.84	6.78	9.22	3.03	9.99	7.92
60 sec	6.22	3.23*	4.57*	.60*	.69*	1.53*
5 min	6.10	1.33*	1.52*	.69*	.90*	.97*

No. of animals indicated in parentheses.

* Magnitude of decrease significantly greater than saline control concentration at each indicated time ($p<.05$).

TABLE III. Mean Plasma Serotonin Concentrations (μg./ml.) in Carotid Artery Blood Following Intravenous Injection of *E. coli* Endotoxin.

Time after Endotoxin		Dose of Endotoxin (mg./Kg.)				
	Saline Control (5)	.01 (5)	.05 (5)	.25 (5)	1.0 (10)	4.0 (10)
Control	.04	.13	.12	.03	.10	.12
15 sec	.05	.07**	.76*	1.25*	2.28*	2.25*
30 "	.07	.15**	.70*	.68*	.93*	1.16*
60 "	.03	.05**	.47*	.22*	.26*	.49*
3 min	.05	.03	.04	.09**	.23**	.38*
5 "	.08	.03	.01	.06	.12**	.28**

No. of animals indicated in parentheses.

* Significantly greater than saline control at each indicated time ($p<.05$).

** Not significantly greater than saline control at each indicated time ($p>.05$).

of 0.01 mg/kg caused no mortality in 15 rabbits, whereas each higher dose produced some mortality, and 4.0 mg/kg was lethal for all rabbits studied.

Discussion. The immediate release of serotonin into plasma following endotoxin injection is strikingly similar to the response of the sensitized rabbit to antigen(6) and correlates well with previously reported *in vitro* release of serotonin after incubation of rabbit blood with endotoxin(12). The lack of a demonstrable rise of plasma serotonin when a non-lethal dose of endotoxin was employed does not imply that serotonin release is responsible for mortality, but shows clearly that massive serotonin release is absent with non-lethal amounts of endotoxin. Serotonin release at a slow rate probably does occur, even with a non-lethal dose, since the fall in serum serotonin was significantly greater ($p<.01$) than the fall in platelet count both 60 seconds and 5 minutes after endotoxin.

Although the release after 15 seconds increased in magnitude between the 0.05 and 1.0 mg/kg doses there was no increase beyond 1.0 mg/kg. The larger dose (4.0 mg/kg) however, produced a more sustained elevation of plasma serotonin since at 60 seconds after endotoxin the plasma concentration in that group was greater than that observed in the group which received 1.0 mg/kg and was the only group in which a significant increase in plasma concentration was observed 3 minutes after endotoxin ($p<.003$). Statistical analysis by rank correlation showed that there was no correlation between the maximal rise of free serotonin and the initial serum serotonin concentration at 3 of the 4 doses used. An inhibitor of serotonin liberation thus may be present *in vivo* as recently demonstrated for histamine an *in vitro* anaphylaxis system(13).

Serotonin release immediately after endotoxin injection in the rabbit may with other vasoactive amines contribute to the initial shock reaction. The lack of further increase in free serotonin concentration between 1.0 mg/kg and 4.0 mg suggests that maximal release occurred with the former dose. Since 4.0 mg/kg is a uniformly lethal dose of endotoxin, but 1.0 mg/kg is not, other factors undoubtedly play a role in the lethal response.

Summary. Within 15 seconds after rapid administration of *E. coli* endotoxin in the rabbit a significant release of serotonin into platelet-free plasma occurs *in vivo*. The magnitude of immediate serotonin release was cor-

related with amount of endotoxin administered, and there was no significant rise of plasma serotonin with a non-lethal dose.

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Hypothermia in Mice Due to Influenza Virus Infection.* (27064)

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Many of the naturally occurring influenzal infections of man are symptomless, and most of the overt infections are characterized by the occurrence of fever without pneumonia. In the mouse, which has been used extensively in studies of the pathogenesis of influenza, the main criteria of infection have been death and pulmonary consolidation. To determine whether the mouse could serve as an experimental model for the less severe infections that usually occur in man, we studied the temperature response of mice to infection with influenza virus.

Hypothermia, rather than fever, proved to be a striking feature of these infections. It was associated with extensive lung lesions. We were unable to produce in mice a satisfactory model for studying the difference between symptomless infection and illness without pulmonary consolidation.

Materials and methods. Mice. Female mice of an inbred strain, BALB/c, 4 weeks old, were used. Three weeks after birth, they were weaned and separated from the males. Twenty serial passages of lungs failed to show evidence of latent pulmonary viruses. Throughout this study, the room temperature in which mice were kept was maintained at 75° to 80°F.

Viruses. Two sublines of the PR8 strain of type A influenza virus were used. They were derived from stock virus which had undergone 8 passages in ferrets, 593 in mice, and 168 in eggs.

An "egg-line" virus was obtained by inoculating 0.1 ml of a 10^{-3} dilution of the stock virus into the allantoic cavity of 10-day embryonated hen's eggs. After 45 hours incubation at 37°C, infected allantoic fluids were pooled and frozen at $-48°$C. One ml of the pool contained $10^{7.5}$ infectious doses (ID_{50}) by the allantois-on-shell technique(1) and 512 HA units of virus.

A "mouse-passaged line" was obtained by 7 successive mouse-to-mouse passages of the stock virus at 3-day intervals. Infected lungs from mice of the 7th passage were removed aseptically and kept frozen intact at $-48°$C until used. Immediately before use, a 10% homogenate was made with Standard Medium(1) by grinding the infected lungs in a mortar with alundum. The homogenate was centrifuged at 2500 rpm for 10 min and the

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supernatant was used as an inoculum.

Virus titration. The allantois-on-shell technique described by Fazekas de St Groth and White(1) was applied. Titers were expressed in terms of 50% infectious dose (ID_{50}) per ml.

Several points should be mentioned in applying this technique: 1) For preparation of the allantois-on-shell pieces, 13-day embryonated eggs were used exclusively. 2) Mycostatin (E. R. Squibb and Sons, N. Y.) added to the Standard Medium in a concentration of 20 units per ml effectively controlled contamination by fungi. 3) Titers obtained by this technique were approximately 10 times lower than those of egg infectivity titration (EID_{50}). 4) In determining the amount of virus in lungs of mice, it was found that the virus titers dropped rapidly when a 10% homogenate of the infected lungs was made immediately after removal from mice and kept frozen at −48°C until titration; however, when the infected lungs were kept frozen intact at −48°C and the homogenate was made just before titration, consistently higher titers were obtained.

Inoculation of mice and scoring of lung lesions. A mouse was anesthetized lightly with ether and 0.05 ml of the inoculum was given intranasally. Standard Medium was used as a diluent for the virus inoculum.

Gross lung lesions were scored by the extent of the pulmonary consolidation seen on the lung surface in relation to total lung surface area, i.e., 4+ lung lesion = complete consolidation, 3+ = consolidation of ¾, 2+ = ½, and 1+ = ¼ of total lung field. A numerical score was recorded for each mouse, and the total lung-lesion score of each group of mice was obtained by calculating a percentage of the total possible score(2).

Measurement of rectal temperature. Rectal temperature was measured with a Thermistemp Telethermometer Model TK (Yellow Springs Instrument Co., Yellow Springs, Ohio). For the measurement, a "small animal probe" was inserted into the rectum to a constant depth of 1 cm. About 20 seconds were necessary to obtain a maximum reading. To avoid possible mechanical damage to the rectum, a glycerine and water mixture (1:1) was used as a lubricant for the tip of the probe. Daily measurement was made between 2 and 3 PM.

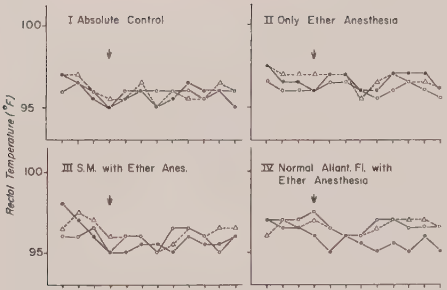
Results. Temperature patterns in control groups. Four groups of 3 randomly chosen mice were put into 4 cages, and rectal temperatures were taken daily. The mice in Group I had no treatment. On the 4th day after grouping, mice in Group II received light ether anesthesia only, those in Group III were inoculated intranasally with Standard Medium under light ether anesthesia, and those in Group IV were similarly inoculated with normal allantoic fluid from 12-day eggs. Fig. 1 shows the results.

There appeared to be a slight fall in rectal temperature of some mice during the first 3 days after initiation of temperature measurement, but thereafter the temperature was fairly stable. None of the 3 treatments (in Groups II, III, and IV) caused a noticeable effect on rectal temperature of mice during the observation period of 8 days after each treatment.

In view of the results of this experiment, rectal temperatures were taken on 4 successive days before mice were used for experiments. The temperature on the 4th day was referred to as the baseline temperature, and was expressed as 0 in the following experiments. A rise or fall in rectal temperature after virus inoculation was indicated as + or − according to the deviation from baseline temperature.

Temperature changes of mice inoculated with egg-line virus. Six groups of 4 mice were used. After measuring baseline temperatures, mice of each group were inoculated with various amounts of egg-line virus. Rectal temperatures were taken daily. On the 7th day after infection surviving mice were killed, and lung lesions were scored. The results are presented in Fig. 2.

It is clear that more rapid and profound hypothermia was observed in the groups inoculated with larger amounts of virus. A fall in rectal temperature was not found in mice of the 10^{-6} group which received a small amount of virus, approximately 2 ID_{50}; and none of the 4 mice in this group had more than 2+ lung lesions. The total lung-lesion score of this group was 35% at end of experiment. One of the mice inoculated with a large amount of virus (see the 3rd mouse in 10^{-2} group) showed no demonstrable hypothermia in contrast to the findings observed in the



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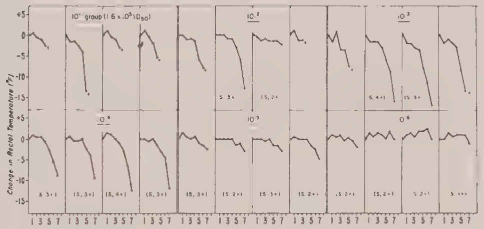

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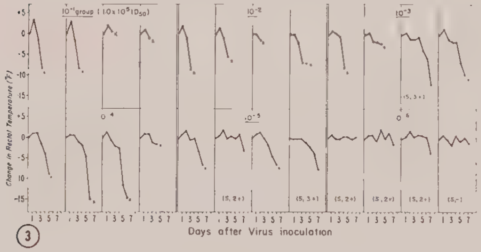

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FIG. 1. Temperature pattern in the control groups of mice. Arrow indicates the time (day) of each treatment. S. M. = Standard Medium. Allant. Fl. = Allantoic fluid.

FIG. 2. Temperature changes of mice inoculated with egg-line of PR8 strain of influenza virus. 10^{-1} group (1.6×10^5 ID_{50}) indicates that mice in this group were inoculated with a 10^{-1} dilution of egg-line virus, and calculated amount of virus inoculum per mouse was 1.6×10^5 ID_{50}. 0 = Baseline temperatures of mice measured before virus inoculation. × = Death. (S,3+) = Survival with 3+ lung lesion at end of experiment.

FIG. 3. Temperature changes of mice inoculated with mouse-passaged line of PR8 strain of influenza virus. See footnotes, Fig. 2.

rest of the mice in this group. The mouse had 2+ lung lesion when killed at end of experiment.

Temperature changes of mice inoculated with mouse-passaged line of virus. In this experiment, mice, grouped as in the preceding experiment, were inoculated with various amounts of mouse-passaged virus. The results are shown in Fig. 3.

As in the preceding experiment with egg-line virus, mice responded to increased amounts of virus inoculum with more rapid development of hypothermia. There was a significant increase in mortality as compared with the preceding experiment. Early death obscured the occurrence of hypothermia in 3 of the mice that received the 10^{-1} and 10^{-2} inocula. The steepness of the temperature curve showing a sharp fall in rectal temperature by 3 days after infection was characteristic of these groups.

In general, it appeared that the mouse-passaged virus caused more rapid development of hypothermia than occurred with egg-line virus. For example, in mice given a 10^{-4} dilution of mouse-passaged virus, hypothermia developed about 24 hours earlier than in those given the same dilution of egg-line virus.

In a subsequent experiment, 3 mice were inoculated with a 10^{-7} dilution of mouse-passaged virus. For a period of 6 weeks their temperatures remained at the level of the baseline temperature even though autopsies made at the end of a 6-week period revealed that they had pulmonary lesions involving ¼ or less of the total lung field.

The course of hypothermia was observed for 6 weeks in a mouse infected with a 10^{-5} dilution of mouse-passaged virus. Its temperature remained at or below the −5 (°F) level for 2 weeks, then gradually returned to baseline temperature. At the end of the 6-week period, the rectal temperature of this mouse was close to the baseline temperature and the mouse appeared healthy. However, it was found at autopsy to have well-defined and markedly constricted lesions involving nearly half of the total lung area.

Relation of hypothermia to amount of virus in the lungs and to extent of pulmonary lesions. To study the relationship of hypothermia as shown in Fig. 2 to virus multiplication in the lungs and to the extent of lung lesions, the following experiments were carried out.

About 40 mice were inoculated with a 10^{-4} dilution of egg-line virus. At various intervals after infection, 3 mice were killed and the lung lesions were scored. The infected lungs were removed aseptically and kept frozen intact at −48°C until all specimens were titrated simultaneously at the end of this experiment. Fig. 4 shows the results.

Following intranasal inoculation, there was no detectable virus in the lungs at 2 and 4 hours. These results constituted a typical lag phase in virus multiplication in the mouse lung as described by Davenport and Francis (3). The virus titer in the lungs reached a maximum level 48 hours after infection. A marked drop in virus titer was observed after the 6th day, presumably due to the action of antibodies produced in the mouse(4).

Lung lesions were seen first on the 2nd day and increased gradually thereafter. The total lung-lesion score was 60% at end of experiment.

From a comparison of the findings presented in Fig. 4 with those in Fig. 2 (see 10^{-4} group), it appears that as a rule hypothermia developed 3 to 4 days after a maximum concentration of virus was present in the lung. Furthermore, hypothermia apparently did not occur in the first several days during which lung lesions were present and relatively limited in extent. Onset of definite hypothermia on the 5th to 6th day corresponded to the time when the total lung-lesion score reached a level of about 50% and was continuing to increase.

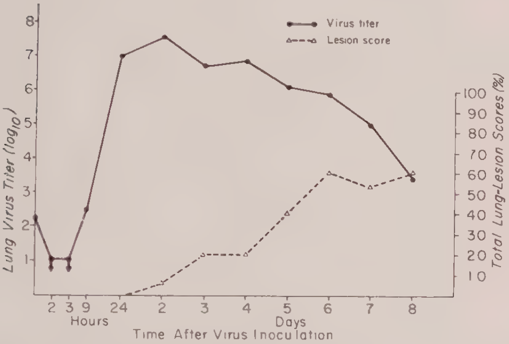

FIG. 4. Virus multiplication and development of lung lesions in mice inoculated with a 10^{-4} dilution of egg-line of PR8 strain of influenza virus. Virus titers were averages of the titers obtained from lungs of 3 mice killed at various intervals after inoculation.

In a similar experiment, mice were inoculated with a lesser amount of the same virus, i.e., a 10^{-6} dilution, which was approximately 2 ID_{50}. At 48 hours after infection the virus titer in the lungs reached the highest level, but in this case the titer was about one-hundredth of that obtained in mice inoculated with a 10^{-4} dilution of the virus. The virus titer remained at this level until the 7th day and then dropped sharply. Lung lesions were seen first on the 3rd day and increased more slowly than in the mice inoculated with a 10^{-4} dilution of the virus. Eventually the total lung-lesion score was about 40% at end of this experiment. As noted in discussing Fig. 2, mice in the 10^{-6} group did not show hypothermia during the experimental period.

Discussion. Among homeothermal animals, the mouse is known to have a relatively poor heat control mechanism. The control mechanism of body temperature may vary from one strain of mice to another as shown by McLaren(5). It has long been known that moderate changes in environmental temperature cause a significant change of body temperature of the mouse(6,7).

Mice have been shown to respond with hypothermia rather than with febrile responses in several kinds of experimental infection(8, 9,10,11). Therefore, it has been suggested that hypothermia is the outstanding characteristic of the thermal response of mice to endotoxins, as well as to a variety of other infectious and toxic agents(9).

In our study it appeared that hypothermia was not related directly to the level of virus multiplication but was related to the extent of pulmonary lesion. In mice inoculated with a 10^{-4} dilution of egg-line virus, hypothermia did not occur until the 5th to 6th day although the virus titer in the lungs was at its maximum level at 48 hours after infection. The initiation of hypothermia coincided with development of pulmonary lesion equal to 50% or more total lung-lesion score.

A transitory febrile response in mice was observed by other investigators in an early stage of some experimental infections which resulted in definite hypothermia in a later stage(9,10). Halberg and Spink(9) have shown that the rise in rectal temperature of mice after administration of *Brucella* endotoxin did not exceed the limit of the "physiological range." In our study, of 24 mice given mouse-passaged virus, 18 mice exhibited an elevation of rectal temperature of at least 1°F at 24 hours after infection, but in all cases the temperature was well within the range observed in uninoculated mice.

Summary and Conclusions. 1. Hypothermia rather than fever was the characteristic thermal response of mice to intranasal inoculation of the PR8 strain of type A influenza virus. 2. Mice showed marked hypothermia with fatal termination in response to inoculation of large amounts, e.g., 1,000 or more infectious doses (ID_{50}), of 2 sublines of the virus, one adapted to embryonated eggs and the other to mice. 3. Inoculation of mice with a small amount of virus, e.g., one to two ID_{50}, did not cause hypothermia. In mice inoculated with about 2 ID_{50} of egg-line virus, the maximum level of virus in the lungs was lower than in mice inoculated with larger amounts of virus, and pulmonary lesions were present but failed to reach the level of 50% total lung-lesion score. 4. In mice inoculated with a moderate amount of egg-line virus, e.g., about 200 ID_{50}, the maximum virus level in the lungs was reached 3 to 4 days before the occurrence of hypothermia. Time of occurrence and degree of hypothermia appeared to be closely related to the extent of pulmonary lesions. 5. The mouse-passaged virus caused more rapid development of hypothermia than the egg-line virus. 6. It is concluded that hypothermia is related to the extent of pulmonary lesion rather than to the extent of virus increase *per se.*

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Neutralization of "Long-Acting Thyroid Stimulator" of Graves' Disease by Antisera to Bovine Pituitary Thyrotropin.* (27065)

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Adams and Purves(1) assayed the serum of patients with Graves' disease for thyrotropin, in guinea pigs given I-131 to label the thyroid and then thyroxine to suppress endogenous release of thyrotropin. They observed the test animal's response to patients' serum to be different from that to standard thyrotropin or to serum from patients with myxedema. Injection of standard thyrotropin or of myxedema serum increased the circulating level of I-131 and a maximum value was reached after a few hours. The level then decreased and was significantly lessened by 16 hours. The serum from Graves' disease on the other hand caused little increase in level early after injection but produced a maximal response at 16 hours or later.

These findings have been amply confirmed in the mouse, similarly prepared for assay purposes except that the responses can be measured at 2 hours and 9 hours after injection(2). The term "long-acting thyroid

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stimulator" (LATS) has recently replaced "serum factor" and "abnormal activator", originally proposed to describe the thyrotropin-like substance in the serum.

Werner and co-workers investigated immunochemically(3) whether LATS is similar or not to human pituitary thyrotropin. Using an antiserum to bovine pituitary thyrotropin, they were able to neutralize the effect on the thyroid of both a human thyrotropin preparation and of a preparation of patients' serum known to contain LATS, made by the alcohol percolation method(4). However, the prepared serum gave only the 2 hour response in the McKenzie mouse assay, so neutralization of the late 9 hour response could not be tested. McKenzie and Fishman(8) then attempted to neutralize the 9 hour response produced by injection of untreated serum from patients with Graves' disease but were unable to do so in 4 trials, although succeeding in neutralizing the 2 hour response to the thyrotropin present in serum from patients with myxedema. They concluded that the serum factor in Graves' disease was not pituitary thyrotropin.

Werner and collaborators had had to use a concentrated globulin from the antiserum to thyrotropin in order to obtain sufficient concentration of antibody to achieve neutralization of LATS, whereas McKenzie and Fishman had used unconcentrated antiserum. It thus appeared likely that the failure of these latter workers could have been due to use of insufficient antibody rather than that LATS was not thyrotropin. Accordingly the problem was reinvestigated except that a globulin concentrate of anti-bovine thyrotropin serum was used as the source of antibody.

Methods. Thyrotropin hormone preparations. The bovine thyrotropin preparation used both as an antigen and as a thyrotropin standard is one in clinical use (Thytropar, Armour). It had a potency of approximately 1 i.u./mg. The human pituitary thyrotropin preparation employed was a relatively crude one made available by Drs. John Bakke and Lawrence Heidemann(3).

Preparation of antisera. Antiserum was developed in rabbits by the methods already reported(5). Six animals were immunized with bovine thyrotropin. An average of 4 i.u. of the thyrotropin was incorporated in 1.0 ml of Freund's adjuvant and injected subcutaneously. Intravenous booster injections were also given until a total of 36 i.u. was given to each rabbit over a period of 2½ months. Ten days after final injection, the animals were exsanguinated under sterile conditions and the sera stored without preservative at 4°C. Globulin fractions of normal serum and antiserum were prepared by sodium sulfate precipitation(6). The volume of the aqueous solutions was reduced to the desired level by dialysis against polyethylene glycol(7), to limit the volume injected intravenously into the test mice.

Bioassay methods. The assay method of McKenzie(2) was employed(3,5) and the tests conducted on the fifth day after I-131 and thyroxine administration. Control blood samples were obtained and the test materials injected intravenously. At 2 and 9 hours after injection, blood samples were again obtained. Radioactivity in the blood samples was measured in an automatic Packard well-type scintillation counter with an average background count of 20 cpm. Groups of 4 mice were employed for each test point. Testing for neutralization of thyrotropin activity was conducted according to reported procedure(5). Antiserum, or the globulin fraction, was mixed with the thyrotropin preparation or serum to be neutralized, placed at 4°C overnight, and then injected without centrifugation the following morning. Since previous absorption of the antiserum with bovine serum to remove anti-bovine serum proteins did not affect the neutralizing potency of the antiserum, absorption was not performed routinely.

Results. Neutralization of beef and human pituitary thyrotropin preparations. The amounts of antiserum required for complete neutralization of both bovine and human pituitary thyrotropin preparations was determined. The findings are summarized in Table 1. Complete neutralization of 0.1 mu bovine thyrotropin preparation was achieved with 0.1 cc of anti-bovine thyrotropin serum, whereas with 0.1 mu human thyrotropin preparation, 0.25 cc of the same antiserum or of an equivalent amount of antiserum globulin was required. Control experiments showed no neutralization by normal rabbit serum or globulin of thyrotropin.

TABLE I. Comparative amounts of antiserum required to neutralize completely bovine and human thyrotropin.

Exp. No.	Test Material	No. of mice	Dose		Blood radioactivity (% of preinjection value as 100%)		P value of difference from value with normal serum or globulin
			Thyrotropin or patient serum mu	Rabbit serum or serum equiv. cc	2 hr Mean ±SD	9 hr Mean ±SD	
383	Saline	4	—	—	84 ± 14	73 ± 19	—
	Bovine thyrotropin (ATSH)	4	.1	—	278 ± 31	194 ± 29	—
	ATSH + Anti-serum	4	.1	.1	133 ± 26	119 ± 31	<.001
	idem	4	.1	.25	125 ± 30	150 ± 23	<.001
397	Saline	4	—	—	124 ± 11	92 ± 31	—
	Human thyrotropin (HTSH)	4	.1	—	247 ± 10	180 ± 17	—
	HTSH + Normal rabbit serum	4	.1	.5	212 ± 60	151 ± 42	—
	HTSH + Anti-serum	4	.1	.1	190 ± 32	155 ± 18	>.1
	idem	4	.1	.25	134 ± 20	145 ± 13	<.01
	idem	4	.1	.5	129 ± 19	107 ± 12	<.001
403	Saline	4	—	—	104 ± 26	81 ± 17	—
	Human thyrotropin	4	.1	—	217 ± 23	122 ± 24	—
	HTSH + Normal Rabbit globulin	4	.1	.25	233 ± 11	188 ± 44	—
	HTSH + Anti-globulin	4	.1	.3	147 ± 22	89 ± 22	<.001
	idem	4	.1	.75	149 ± 9	80 ± 3	<.001

TABLE II. Partial neutralization of Graves' disease serum factor by small amounts of anti-bovine thyrotropin serum.

Exp. No.	Patient	Test Material	No. of mice	Dose		Blood radioactivity (% of preinjection value as 100%)		P value of difference from value with normal serum or globulin
				Thyrotropin or patient serum	Rabbit serum or serum equiv. cc	2 hr Mean ± SD	9 hr Mean ± SD	
387	L.L.	Saline	4	—	—	93 ± 24	92 ± 18	—
		Bovine thyrotropin	4	.1mu	—	228 ± 22	177 ± 86	—
		Serum	4	.1cc	—	261 ± 36	536 ± 41	—
		Serum + Normal Rabbit serum	4	.1 “	.4	302 ± 38	632 ± 147	—
		Serum + Anti-serum	4	.1 “	.4	329 ± 41	519 ± 34	<.1
294	R.H.	Saline	4	—	—	78 ± 12	79 ± 9	—
		Bovine thyrotropin	4	.1mu	—	164 ± 44	151 ± 56	—
		Serum	4	.25cc	—	70 ± 20	241 ± 12	—
		Serum + Normal Rabbit globulin	4	.25 “	1.0	171 ± 26	286 ± 36	—
		Serum + Anti-serum	4	.25 “	1.0	122 ± 47	171 ± 29	<.001
334	R.H.	Saline	4	—	—	105 ± 27	136 ± 21	—
		Bovine thyrotropin	4	.5mu	—	226 ± 9	171 ± 5	—
		Serum	4	.5cc	—	141 ± 5	211 ± 18	—
		Serum + Normal Rabbit globulin	4	.5 “	1.0	326 ± 49	463 ± 66	—
		Serum + Anti-globulin serum	4	.5 “	1.0	281 ± 74	338 ± 50	<.001
420	R.H.	Saline	4	—	—	100 ± 13	100 ± 13	—
		Human thyrotropin	4	.1mu	—	343 ± 73	286 ± 64	—
		Serum	4	.2cc	—	248 ± 44	364 ± 109	—
		Serum + Normal Rabbit globulin	4	.2 “	1.0	282 ± 128	343 ± 152	—
		Serum + Anti-globulin	4	.2 “	1.2	183 ± 20	240 ± 53	<.1

Neutralization of LATS of Graves' disease. Ten experiments were conducted with serum from each of 6 patients with Graves' disease (Tables 2 and 3). Five of the 6 had severe eye changes, the sixth had no ocular manifestations. Amounts of antisera or corresponding amounts of globulin employed varied from 0.4 cc to 2.5 cc per mouse. These were mixed before assay with the minimum amount of patient's serum capable of giving an unequivocal 9 hour assay response. Control studies were made concurrently.

TABLE III. Complete neutralization of Graves' disease serum factor by large amounts of antibovine thyrotropin serum.

Exp. No.	Patient	Test Material	No. of mice	Dose		Blood radioactivity (% of preinjection value as 100%)		P value of difference from value with normal serum or globulin
				Thyrotropin or patient serum	Rabbit serum or serum equiv. cc	2 hr Mean ± SD	9 hr Mean ± SD	
452	G.C.	Saline	4	—	—	124 ± 10	123 ± 27	—
		Bovine thyrotropin	4	.1mu	—	328 ± 15	251 ± 39	—
		Serum	4	.1cc	—	174 ± 42	311 ± 63	—
		Serum + Normal Rabbit globulin	4	.1 "	2.0	177 ± 9	269 ± 73	—
		Serum + Anti-globulin (unabsor)	4	.1 "	2.0	147 ± 34	132 ± 18	$<.001$
		Serum + Anti-globulin (absor)	4	.1 "	2.0	132 ± 4	116 ± 7	$<.001$
438	L.W.	Saline	4	—	—	106 ± 10	87 ± 10	—
		Bovine thyrotropin	4	.1mu	—	189 ± 8	155 ± 18	—
		Serum	4	.05cc	—	171 ± 53	223 ± 129	—
		Serum + Normal Rabbit globulin	4	.05 "	2.5	131 ± 16	219 ± 43	—
		Serum + Anti-globulin	4	.05 "	2.5	123 ± 18	134 ± 18	$<.001$
434	B.J.	Saline	4	—	—	85 ± 31	83 ± 16	—
		Bovine thyrotropin	4	.1mu	—	386 ± 81	238 ± 86	—
		Serum	4	.1cc	—	151 ± 14	214 ± 34	—
		Serum + Normal Rabbit globulin	4	.1 "	2.5	175 ± 34	217 ± 24	—
		Serum + Anti-globulin	4	.1 "	2.5	142 ± 42	145 ± 67	.01
419	B.J.	Saline	4	—	—	80 ± 8	63 ± 23	—
		Human thyrotropin	4	.1mu	—	261 ± 31	190 ± 17	—
		Serum	4	.1cc	—	124 ± 16	201 ± 27	—
		Serum + Normal Rabbit globulin	4	.1 "	2.5	148 ± 12	211 ± 22	—
		Serum + Anti-globulin	4	.1 "	2.5	121 ± 34	149 ± 41	$<.001$
413	L.L.	Saline	4	—	—	113 ± 5	110 ± 17	—
		Bovine thyrotropin	4	.1mu	—	240 ± 34	194 ± 30	—
		Serum	4	.05cc	—	134 ± 15	199 ± 26	—
		Serum + Normal Rabbit globulin	4	.05 "	2.5	157 ± 34	214 ± 47	—
		Serum + Anti-globulin	4	.05 "	2.5	122 ± 23	124 ± 22	$<.001$

Inadequate amounts of antiserum gave partial neutralization of the LATS in patients' sera (Table 2). However, complete neutralization was achieved with larger amounts of antiserum in all of 5 experiments, and the I-131 level in the serum of the test mice failed to rise significantly above pre-injection value at either 2 or 9 hours after injection (Table 3). Specificity of the neutralization reaction was confirmed by the demonstration that neither preliminary absorption of the antiserum with bovine serum nor incubation with normal rabbit serum globulin caused neutralization of LATS.

Discussion. The successful neutralization of LATS in the serum of patients with Graves' disease accomplished with antibody to bovine thyrotropin confirms the previous findings of this laboratory(3). To achieve neutralization of a minimally effective amount of LATS, antibody from 2.5 cc of antiserum was required. This amount is far in excess of that used by McKenzie and Fishman(8) and undoubtedly explains their failure.

The ability of antiserum to thyrotropin to neutralize LATS suggests several possibilities as to its nature: 1) that it is circulating human pituitary thyrotropin with another substance or substances in the patients' serum accounting for both the long-acting effect and the large doses of antibody needed for neutralization; 2) that it is circulating human pituitary thyrotropin but is bound differently to carrier proteins than in the normal state; or 3) that it is different from human pituitary thyrotropin but has sufficient determinants in common so that it can cross-react with, and be neutralized by, antithyrotropin sera.

About 2.5 times as much anti-bovine thyrotropin serum was required to neutralize the same amount of standard human pituitary preparation as standard bovine hormone material. About 10 times the amount of antiserum to neutralize human thyrotropin was necessary to neutralize the effect of an approximately equivalent amount of LATS. Unfortunately this latter fact does not help distinguish among the speculations listed above as to the nature of LATS.

Summary. A globulin concentrate from antiserum to bovine pituitary thyrotropin completly neutralized the effect on the thyroid of "long-acting thyroid stimulator" in the serum of patients with Graves' disease. Both early and late responses in the McKenzie assay were abolished. A globulin concentrate from normal rabbit serum had no neutralizing effect. About 10 times as much antibody was required to neutralize LATS as the approximately equivalent potency of human pituitary thyrotropin.

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Effect of Vitamin A Deficiency on Taste.† (27066)

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Vitamin A is necessary for maintaining the integrity of epithelial tissue. Since taste buds consist of modified epithelial cells, a role for vit. A in the taste mechanism readily suggests itself. It was recently reported that vit. A-deficient rats were apparently unable to taste quinine and saccharin(1). The present study explored this phenomenon using quinine sulphate and sodium chloride over an extended period of time, and tested the effect of administration of vit. A on recovery of normal taste function.

Materials and Methods. Eleven 6-week old albino rats (CFN, Carworth) were placed on the USP vit. A-deficient diet. Twenty four-hour, 2-choice preference trials began 14 weeks later, when the rats were still growing normally. The animals were in individual cages, fitted with 2 Richter-type graduated drinking tubes, one tube containing distilled water and the other, a test solution. Measurements were made to the nearest ml and the tubes were rinsed and filled with fresh solution every day. Percent preference was calculated as ml of test solution drunk, divided by ml of total fluid consumed, times 100. When total intake was less than 5 ml, the preference was not calculated. This criterion eliminated 13 readings out of 2800. A group of 9 normal rats received the same test solutions, and another group of 6 normal rats were given water in both tubes (H_2O control). The controls were subject to the same conditions as the experimentals. The right-left position of the tube containing the test solution was changed daily according to a restricted random schedule. Preference tests were divided into 8-day periods, during which each chemical was presented randomly for 4 days and appeared equally often in each position. The rats had a choice between either NaCl or quinine sulphate (QSO_4) and distilled water for 24 hours. Pre-

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ferences given in this paper are the group means for the 4 days.

Results. A decrease in degree of rejection of 8 μM QSO_4 solution occurred in the terminal stages of vit. A depletion (Fig. 1A). All the deficient-group mean QSO_4 preferences, except those for 147 and 155 days on diet, are significantly greater than the normal group preferences for the 7 periods shown on the graph. P values range from < 0.0003 to 0.04; unless otherwise noted, this and subsequent p values are one-tailed and based on the Mann-Whitney U test(2).

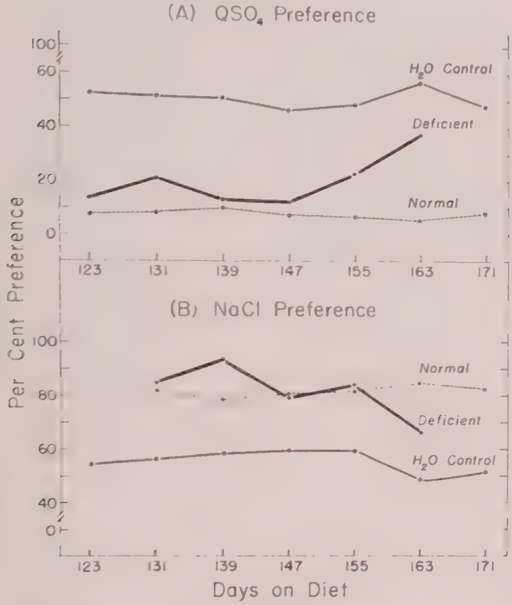

FIG.1(A). Preference functions of normal and vit. A-deficient rats for 8 μM quinine sulphate (QSO_4) solution.

(B). Preference functions of the same rats for .05 (131 days), .02 (139 days), .01 (147 days,) and .008 M (155-163 days) NaCl solutions. Preference of H_2O controls is not significantly different in either case from an ideal 50% choice ($p=.71$; $p=.26$; 2-tailed).

Prior to 8 μM QSO, the rats were exposed to 40, 20, and 10 μM solutions, each for one full period. Significant differences appeared at 20 and 10 μM ($p<0.025$, $p<0.001$). At this time the rats were still growing normally. Weight losses began 16 days later, after 123 days on the diet.

Figure 1B shows the change in NaCl preference over the same 7 periods. Concentration was decreased in each period until 0.008 M was reached at 155 days. As was the case for quinine rejection, NaCl selection was significantly lower than the normals ($p<0.025$) toward the end of the depletion period. In fact, the deficient group was not significantly different from the H_2O controls ($p=0.08$). The deficient group showed a significantly higher preference for 0.02 M NaCl at 139 days ($p=0.002$, 2-tailed). The reason for the increased selection is not apparent.

Deaths occurred from secondary infections after 139 days on the diet. The last 3 means on both graphs represent 9, 7, and 5 rats respectively. During these 3 periods the deficient rats were regularly given antibiotics intramuscularly. Control rats were given an initial dose only. Four rats developed xerophthalmia and were given vit. A intraperitoneally. Two recovered.

Vit. A was given on an individual basis and the treated animals' preferences were not included with those of the non-treated. Preferences of the non-treated animals continued in the direction of indifference (50%), but because of the reduced group size, they are not plotted on the graphs.

The 2 surviving rats were studied for 6 and 8 weeks respectively after administration of vit. A. At the end of the period they had regained their weight and appeared normal. Table I shows that the treated rats differed significantly from the controls in QSO_4 preference. They did not differ significantly in preference for NaCl. Moreover, the normal NaCl preference was reestablished immediately following treatment. Mean preference of the rats in the 2 periods preceding vit. A treatment are listed for comparison.

TABLE I. Effect of Vit A on Mean Preferences of 2 Vit A Deficient Rats for QSO_4 and NaCl Solutions.

	QSO_4	NaCl
Pre-vit A	45.1	58.0
Post-vit A	47.2*	82.2‡
Control Rats	8.7	80.3

* $P<.003$ ‡ $P = .57$ (both two-tailed.)

Discussion. These results indicate that vit. A is required for normal taste function. Dowling and Wald(3) recently reported that rat liver stores and blood levels of vit. A dropped to zero 2 to 3 weeks before weight losses began. At the same time, the rhodopsin level declined and the light threshold for the electroretinogram began to increase. The first significant change in QSO_4 rejection by our rats occurred in the third week preceding weight losses, which would correspond to the time when the supply of vit. A was completely depleted. However, since thresholds were not measured, changes may have occurred earlier. The late appearance of changes in NaCl selection and the lack of recovery of QSO_4 rejection suggests the possibility of different mechanisms underlying gustatory stimulation.

The rapid recovery of NaCl selection tends to rule out peripheral nerve damage or physical blocking of the taste pore as the reason for the taste deficit. A direct effect on the taste cells may reasonably be hypothesized. Vit. A may thus play a role in the functional and/or structural integrity of the taste cells. Verification will require histological and electrophysiological studies.

Further, the effect of vit. A may be specific or merely the result of a general effect on epithelial tissue. Dowling and Wald(4) reported that vit. A acid maintained normal growth in rats but did not prevent development of night blindness, thus limiting the role of vit. A to the visual system. An experiment is now underway in our laboratory to determine whether vit. A acid may also fail to prevent development of a taste deficit. If this is the case, vit. A would be implicated as a specific requirement in taste as in vision.

Summary. Rats in the advanced stages of vit. A depletion showed abnormal taste responses. A marked decrease in rejection of quinine and selection of NaCl solutions occurred. Administration of vit. A resulted in rapid recovery of NaCl selection but not of quinine rejection. These data suggest that vit. A may have a direct effect on the functioning of the taste cells.

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Effect of Estrogen and Estrogen-like Compounds on Atherosclerosis in Cholesterol Fed Cockerels. (27067)

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Exogenous sex hormones have been observed to modify coronary atherosclerosis in experimental animals(1). Feminizing doses of estradiol benzoate will inhibit coronary atherosclerosis in cholesterol fed cockerels and will induce regression of pre-existing lesions. The mechanism of estrogen inhibition of coronary atherosclerosis is as yet unclear, though data suggest a relationship with its feminizing potency(2). Three estrogen-like compounds of low feminizing potency,* synthesized and purified by Dr. Paul Keurath, were used to study further the relationship between these effects. Two of these analogues did have an effect on the course of the experimental disease.

Methods. Forty white leghorn cockerels, one month of age, were divided into 6 experimental groups, and each bird was allocated to an individual cage. All birds received a basic diet of standard chick growing mash. The experimental diet was formulated by supplementing the growing mash with 1% cholesterol and 20% cottonseed oil. The 3 estrogen-like analogues, 2 iodo-estradiol, 2 iodoestrone and 2 iodo-estrone acetate, all of low feminizing potency,* were injected subcutaneously in 0.5 mg doses every other day. Estradiol benzoate was given similarly in 1 mg doses.

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* Feminizing effects of these compounds were bioassayed in ovariectomized rats by the Endocrine Laboratories, Madison, Wisc.

At the end of 3 months on this regimen, the birds were sacrificed, weighed, and their combs and wattles measured and testes weighed. Autopsies were performed and tissues submitted for histologic eamination. The aortas were opened longitudinally and fixed in troughs of formalin. The hearts were fixed whole, and 5 transverse blocks were cut from each for histologic examination. All sections were stained with H & E and selected sections with oil red O to demonstrate fat.

The minimum histologic criterion for atherosclerosis was arbitrarily established as subendothelial vacuolization with elevation of the endothelial cells by accumulated fat. The total number of arteries was counted and recorded according to size (large, medium, and small) and degree of involvement assessed. Arteries of the right and left ventricle were recorded separately.

Results. The effects of the diet on growth and the feminizing effects of estradiol benzoate and the estrogen analogues were estimated by determining body weight, comb dimensions, testicular weight, and spermatogenesis and by calculating testicular weight/body weight ratios. Table 1 illustrates the effects observed in experimental birds and controls.

Table 2 is a record of the atherosclerosis observed in coronary arteries and aorta. The coronary atherosclerosis induced by the fat and cholesterol feeding involved predominately the small arteries in the subendocardial one third of the left ventricle. The vessels of the right ventricle were spared. In Table 2 it is seen that the 2 observers varied in their interpretation of how frequently atherosclerosis was present in coronary vasculature, but the variation was not so great but that clear differences could be distinguished between some of the groups. As expected, the animals given estradiol benzoate had a significantly reduced incidence of coronary atherosclerosis. The compounds 2 iodo-estradiol and 2 iodo-estrone

TABLE I. The means of measurements of primary and secondary sexual structures are recorded and indicate effect of diet and chemical compounds on sexual development.

Groups	Comb Length (cm)	Comb Height (cm)	Wattle Length (cm)	TW/BW* x 10^{-4}
Untreated	12.7	8.0	6.3	70
Cholesterol	10.1	6.3	4.7	50
Cholesterol and estradiol benzoate	5.6	3.0	1.3	5
Cholesterol and 2-iodo-estradiol	10.2	6.4	4.6	42
Cholesterol and 2-iodo-estrone	9.5	6.0	4.0	33
Cholesterol and 2-iodo-estrone acetate	9.2	5.7	3.7	25
Standard error of any mean in the column above	0.5	0.4	0.1	10

* TW/BW is abbreviation for testicular wt/body wt ratio.

TABLE II. Degree of atherosclerosis in both coronary arteries and aorta is tabulated to demonstrate effects of diet and chemical compounds.

	No. of arteries counted	Coronary Arteries % of coronary arteries showing atherosclerosis		Aorta % of surface involved by atherosclerosis Mean of 2 observers' scores*
		Observer 1	Observer 2	
Untreated	—	0	0	7.0
Cholesterol	181	17.9	21.1	15.7
Cholesterol and estradiol benzoate	161	6.1	6.9	61.0
Cholesterol and 2-iodo-estradiol	168	14.4	18.6	19.0
Cholesterol and 2-iodo-estrone	190	17.1	21.1	30.4
Cholesterol and 2-iodo-estrone acetate	174	12.3	12.5	18.0
Standard error of any mean in column above	5.7	2.9	3.7	0.6

* Mean of 2 observers' scores is reported since the difference between the 2 was not significant.

had no effect on the atherosclerosis when compared with the effect of the cholesterol diet. The effect of 2 iodo-estrone acetate just failed to reach a statistical difference of P = .05 when compared with the other 2 analogues and with estradiol-benzoate. The statistical analysis was made by the method of Duncan (3). However, the effect of 2 iodo-estrone acetate lies between that of the other 2 analogues and that of estradiol-benzoate.

In contrast to the protective effect of estradiol benzoate on coronary vessels, the effect on the aorta was definitely to enhance atherosclerosis. Of the 3 analogues tested, only 2 iodo-estrone had a statistically significant enhancing effect on aortic atherosclerosis (P = .05), but the effect was less marked than that of estradiol. The other 2 analogues had no greater effect than the cholesterol diet alone.

Discussion. In this experiment estradiol benzoate produced feminization, inhibition of coronary atherosclerosis and enhancement of aortic atherosclerosis similar to reports previously made by others(4,5) and thus acted as a positive control. When the effects of the 3 estrogen analogues are compared with those of estradiol benzoate and with the cholesterol diet alone, several interesting differences are observed. The effects of the analogues on the coronary arteries did not reveal statistically significant protection by any of the 3. However, the effect of 2 iodo-estrone acetate lies between the other 2 analogues on the one hand and estradiol benzoate on the other. Due to the lack of statistical significance in either direction, this result is not clear-cut, but suggests a difference between 2 iodo-estrone acetate and both estradiol benzoate and the other analogues.

In contrast to the observations on the coronary arteries, estradiol benzoate had a distinctly enhancing effect on aortic atherosclerosis. Similarly 2 iodo-estrone had a statistically significant effect in enhancing aortic atherosclerosis. This effect of 2 iodo-estrone was not so great as estradiol benzoate, but was definitely greater than the cholesterol diet alone. The other 2 analogues had no effect on the aortic lesions.

Comparison of the feminizing effects of the 3 analogues reveals 2 iodo-estradiol to have no identifiable effects over those general growth effects of the experimental diet alone. The other 2 analogues appear to have slight feminizing capacity.

When the above effects are correlated, it is seen that 2 iodo-estrone acetate resembles estradiol benzoate more closely than the other 2 analogues in its effects on coronary atherosclerosis whereas 2 iodo-estrone most closely resembles estradiol benzoate in its effect on aortic atherosclerosis. Since these two compounds have only limited feminizing effect and yet differ definitely from one another in their effects on coronary arteries and aorta, it is suggested that the effects of these compounds on coronary atherosclerosis and on aortic atherosclerosis are mediated by differences in molecular configuration other than those inducing feminization. Further, it is suggested that the differences in molecular configuration between 2 iodo-estrone and 2 iodo-estrone acetate are manifested by differences in their comparative activities on the aorta and coronary arteries.

Summary. Coronary and aortic atherosclerosis were studied in white leghorn cockerels fed an atherogenic diet and treated with parenteral estradiol benzoate or synthetic estrogen compounds of lower feminizing potency. The estradiol benzoate gave the expected feminization and protection against coronary atherosclerosis and enhanced thoracic aortic atherosclerosis. The 2 iodo-estrone acetate gave some suggested protection to the coronary vasculature, but was not clearly different from the other analogues in this respect. The 2 iodo-estrone enhanced aortic atherosclerosis and was significantly different in this effect from the other analogues. These observations suggest that the effects on atherosclerosis of the coronary arteries and aorta are mediated through molecular configurations different from those mediating feminization.

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Time Course of Thyroid Response to Rising and to Falling Blood Levels of Thyroxine.*† (27068)

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Feedback control of pituitary thyrotrophin release by thyroxine has been amply demonstrated (cf.1). Although the inhibitory effect of thyroxine may be exerted in part through the hypothalamus(2), there is good evidence to show that this hormone regulates TSH output primarily by a direct action on pituitary tissue (cf.3). Little is known of the relationship between blood thyroxine concentration and rate of TSH secretion; still more obscure is the cellular mechanism by which thyroxine exerts its regulatory effects on the pituitary. In an attempt to define this problem more precisely, experiments to determine the time course of pituitary-thyroid response to increase in thyroxine concentration and to falling blood thyroxine levels were carried out.

Methods. This study was done in two parts. The first, utilizing rabbits, was carried out several years ago and the second experiment, utilizing rats, was more recently completed.

A. *Inhibitory response to thyroxine injection.* The technique for measuring thyroid activity in the rabbit and conditions of housing and diet have been recorded(4). In the present experiment 3 rabbits were used to measure accurately the latent period of thyroid inhibition following intravenous thyroxine injection. Twice daily neck counts of thyroid radioactivity were made beginning 48 hours after intraperitoneal injection of 40 μc of NaI^{131}. From the time of the first count 20 mg of methyl thiouracil were administered subcutaneously twice daily to block the uptake and reutilization of radioiodine from degraded endogenous I^{131}-labeled thyroxine. The use of the goitrogen also caused a marked acceleration of thyroid I^{131} release rate which permitted greater precision in measurement of the time at which thyroid inhibition occurred. Following an initial observation period of 3 days, 250 μg of l-thyroxine dissolved in 2 ml of slightly alkaline physiological saline were injected intravenously at an accurately noted time. Five additional neck counts were obtained over the next 3 days. The time of onset of inhibition was estimated from the intersection of the control release curve with the backward extrapolated curve of the thyroxine period (Fig. 1). Continued thyroidal inhibition was ensured by giving an additional dose of 100 μg of thyroxine one day later.

B. *Release of thyroid inhibition as blood thyroxine levels fall.* The principle of this experiment was to follow serially plasma PBI levels as they fell after thyroxine injection, and to correlate these values with measurements of thyroid gland activation as determined by serial changes in plasma PBI^{131}.

Male Holtzman rats of the Sprague-Dawley strain, 250 to 400 g in weight were used for this experiment. They were housed individually in a controlled temperature room and were fed Purina Laboratory Chow *ad libitum.* On the first day of experiment the rats were injected with 20 μc of NaI^{131} i.p. Exactly one day later, 20 μg of l-thyroxine were similarly injected. At intervals thereafter groups of 8 rats each were sacrificed under ether anesthesia by bleeding from the carotid artery through a polyethylene cannula into heparinized tubes. Chemical PBI was determined by the method of Chaney, *et al.*(5) on plasma from individual rats. Total and 10% trichloracetic acid precipitable radioactivity were determined in a scintillation well counter. Appropriate corrections for radioactivity decay were made.

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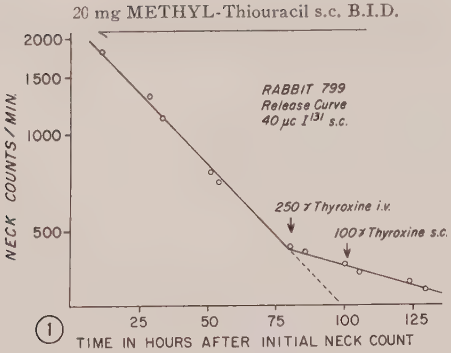

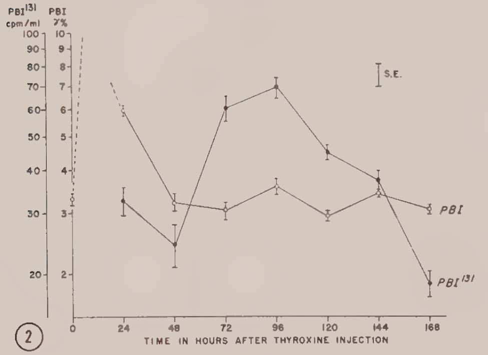

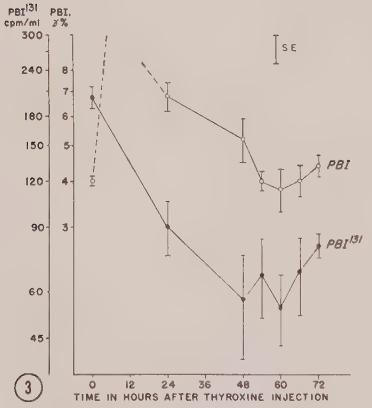

FIG. 1. Effect of intravenous injection of thyroxine on I^{131} release from the thyroid gland of a rabbit measured by external counting. Methyl-thiouracil was injected to prevent I^{131} recirculation and to increase rate of activity of pituitary-thyroid axis. Time of inhibition is coincident with time of injection.

FIG. 2. Change in blood protein bound iodine (PBI) and trichloracetic acid insoluble plasma radioactivity (PBI^{131}) which follows injection of 20 µg l-thyroxine i.p. in groups of rats (8 rats per time interval) pre-treated with NaI^{131}. Radioiodine was injected 24 hr before start of experiment. At 0 time thyroxine was injected and between 24 and 48 hours PBI continued to fall, leveling off between 48 and 72 hours. Marked activation of pituitary-thyroid axis occurred betwen 48 and 72 hours as measured by concentration of PBI^{131}.

Results. A. Latent period of thyroid inhibition following thyroxine injection. Following intravenous injection of l-thyroxine, a prompt slowing of release of I^{131} from the thyroid gland occurred in each of the 3 rabbits (Fig. 1). Release rates fell in the 3 rabbits respectively from 41 to 8% per day, from 64 to 15% per day and from 39 to 13% per day. The time of onset of the inhibitory effect was estimated from the interception of the pre- and post-injection slopes. From these intercepts the onset of inhibition of release was observed to be coincident with time of thyroxine injection in 2 rabbits and 3 hours after injection in the third rabbit.

B. *Release of thyroid inhibition as thyroxine levels in the blood fall.* In the first group of rats studied observations were made at 24 hour intervals for 7 days after injection of thyroxine (Fig. 2). Mean plasma PBI had fallen to control levels by the 48 hour specimen and remained at control levels over the next 5 days. Plasma precipitable radioactivity which reflects the release of preformed I^{131}-labeled l-thyroxine was still falling between 24 and 48 hours. Between 48 and 72 hours (at a time when falling PBI concentration had leveled out), there was a sharp increase in plasma protein bound radioactivity, indicating thyroid activation. The interval 48 to 72 hours was explored in more detail in a second experiment by making measurements every 6 hours (Fig. 3). PBI concentration fell up to 54 hours after thyroxine injection, then leveled out sharply at values not significantly different than the normal. Thyroid activation as measured by PBI^{131} increase occurred prior to time of inflection of the curve of PBI concentration, i.e., between 48 and 54 hours which corresponds to plasma PBI levels falling from 5.2 µg% to 4.0 µg%.

Discussion. The brief duration of the latent period of pituitary-thyroid responses to al-

FIG. 3. Change in blood protein bound iodine (PBI) and trichloracetic acid insoluble plasma radioactivity (PBI^{131}) which follows injection of 20 µg of l-thyroxine i.p. in groups of rats (8 rats per time interval) pre-treated with NaI^{131}. Radioiodine was injected 24 hr before start of experiment. Falling PBI level stabilizes between 54 and 60 hours and activation of pituitary-thyroid system occurs between 48 and 54 hours when PBI values are falling from 5.2 to 4.0 µg%.

tered blood thyroid hormone levels has been brought out in these experiments and emphasizes the point made previously that this system is not as sluggish as traditionally considered(4). The latent period of thyroid inhibition following thyroxine injection involves 2 components: inhibition of TSH release and the dying away of thyroid stimulation from TSH already present in the blood. The latter time has been measured in the rabbit(6), and a "half-time" of TSH effect on I^{131} release of 70-100 minutes determined. In this experiment times of onset of thyroid inhibition no longer than 3 hours were observed. Considering the lag before TSH effects on I^{131} release wear off, it must be concluded that thyroxine acts to stop TSH secretion within one hour, and probably within a few minutes after injection. A direct effect of thyroxine on the thyroid gland can be excluded by previous studies in the rabbit(7).

In the second group of experiments plasma PBI has been taken as a measure of plasma thyroxine concentration even though it is highly likely that it is the free thyroxine level (8), and not the bound which is responsible for the physiological effects on the pituitary. Nonetheless, the bound form is in equilibrium with the free; changes in PBI will reflect changes in the physiologically active hormone. Response to falling thyroxine concentration is governed by the critical level of active hormone which triggers TSH secretion. Components in the measured change in thyroid activation are the latent period of the pituitary itself and the time it takes for TSH to elicit a measurable change in plasma radioactivity. Recent studies of Söderberg(9) indicate that bound I^{131} discharge after TSH can be detected in thyroid venous blood within 15 minutes(1). The thyroid lag therefore is quite short. Neither the critical thyroxine concentration nor the pituitary activation lag are known however, and their calculation in the present experiment is not possible because one or the other datum is essential for the precise interpretation of the curves. It is reasonable to conclude, however, that TSH release is activated as the PBI is falling from 5.2 to the normal level of 4.0 $\mu g\%$, and that the critical level for activation probably lies between these values. Reinstitution of TSH secretion is probably accomplished within 6 hours after the critical thyroxine concentration is achieved.

Summary. In a study of the relationship between blood thyroxine concentration and thyrotrophin (TSH) secretion, the time course of thyroid response to thyroxine injection has been examined. As measured by thyroid I^{131} release curves pituitary-thyroid inhibition occurred immediately following sudden increase in blood thyroxine concentration in 2 rabbits and within 3 hours in a third. Groups of rats were pre-treated with radioiodine and thyroid inhibition produced by injection of thyroxine. Protein bound iodine (PBI) levels in the blood were followed serially as the inhibitory effects of the injected material gradually decreased. Pituitary-thyroid activation (as measured by sharp increase in PBI^{131}) was observed as PBI levels fell from 5.2 to 4.0 $\mu g\%$ (control PBI 4.0 $\mu g\%$) over a 6-hour period.

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Failure of Orchiectomy to Affect Degenerative Joint Disease in STR/1N Mice. (27069)

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Osteoarthritis is a genetically governed disease in laboratory mice. Among several endocrine or metabolic parameters studied that might conceivably have a major influence on the genesis of the lesions, only one, in our hands, has proved important: spontaneous degenerative joint disease is more frequent in male than in female mice(1). Other investigators have found that orchiectomy, performed early in life, markedly reduced the occurrence of joint lesions in C57 black and DBA mice(2); administration of 17-ethyl-19-nortestosterone largely reversed this finding (3). Furthermore, although bone length was not measured, histologic findings suggested that epiphyseal growth was retarded by orchiectomy in mice. During the course of other studies, it was surprising to note that neither the reported diminution of osteoarthritis nor retardation of bone growth occurred following early castration in STR/1N mice, a strain particularly prone to this disorder. For this reason, it seemed worthwhile to place these findings on record.

Methods and materials. Five groups of STR/1N mice were raised until 16 months of age: 1) non-breeder males; 2) sexually active males; 3) males orchiectomized at 4-6 weeks of age; 4) breeder females and 5) virgins. They were housed concurrently in groups of 4-6, usually 5, and fed Purina laboratory chow. The uneven numbers of males (Table 1) resulted from an intercurrent uropathy to which intact males of the strain are susceptible. Skeletons were prepared by maceration with papain; osteoarthritis of the knees, evaluated on a 0 to 4 severity scale, and femur lengths were measured as in previous studies(4). For reasons discussed elsewhere(4), the data concerning the joint disease were tested statistically by the ridit method of Bross(5), rather than by conventional procedures applicable to continuous values following a normal distribution.

Results. The frequency and severity of osteoarthritis of the knees were significantly (p. <.05) less in females than in males (Table 1). Breeding was without influence on the joints in females; in breeder males, the median osteoarthritis score was somewhat less than in non-breeders (Fig. 1) although the signifi-

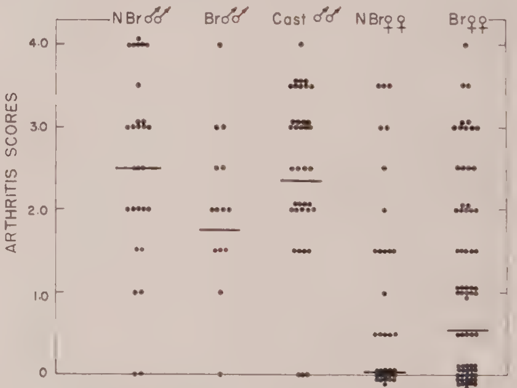

FIG. 1. Distribution of osteoarthritis scores of each knee in 5 groups of STR/1N mice. Horizontal lines represent median score for each group. Br: breeder; N Br: non-breeder; Cast: castrate.

TABLE I. Osteoarthritis, Femur Length and Body Weight in STR/1N Mice, 16 months old.

Group—(No. mice)	Osteoarthritis (Ridits)*			Femur Length‡ (mm)	Final Body‡ Wt (g)
	Mean	Upper CL	Lower CL		
♂ ♂ Non-breeders—(14)	.498†	—	—	15.86 ± .09	44.4 ± .9
♂ ♂ Breeders—(7)	.352	.487	.217	15.65 ± .09	41.9 ± 1.7
♂ ♂ Castrate—(19)	.474	.554	.394	16.27 ± .05	53.2 ± 1.1
♀ ♀ Non-breeders—(17)	.183	.264	.102	15.69 ± .05	45.4 ± 1.6
♀ ♀ Breeders—(34)	.207	.259	.155	15.81 ± .04	47.3 ± 1.2

* Both knees; CL: 95% confidence limits.

‡ Mean ± standard error.

† The identifiable distribution to which no confidence limits are attached.

cance of this finding is questionable in view of the small number of animals in this group. Castrate males had articular lesions as often and severe as intact males. They were significantly (p. <.01) larger (mean femur lengths) and heavier than were the intact males.

Discussion. It is difficult to interpret the failure of orchiectomy to reduce the amount of joint disease in STR/1N mice in view of different findings by others already noted. It is conceivable that strain differences have an important role in determining the effect of the testes on osteoarthritis. The apparent arthritis-sparing influence of femaleness in STR/1N mice was not simulated by removal of the testes; presumably an ovarian contribution must be involved in the sex difference.

The increased length of the bones in the castrate mice does not lend support to the view (3) that the growth-promoting effects of testicular or other hormones have an important enhancing effect on degenerative joint disease in mice. Castration prevented the obstructive uropathy already referred to. This lesion was absent from the intact animals that survived for 16 months and so were included in this study. For this reason, any debility attending the uropathy can be excluded as a contributory factor to the bone growth or articular findings. The greater obesity of castrate than intact males probably does not account for the failure of orchiectomy to reduce the amount of joint disease. Not only are the STR/1N females as obese as the males, but other studies (4) on males of several strains, including STR/1N, have shown that obesity *per se* does not have deleterious effects on the joints of mice. The relatively lower body weights in intact males than in females in the present experiment, on the basis of past experience, is the result of a decline from peak values that occurs in this strain in the second year of life. Femur lengths were comparable in intact animals of both sexes. We have found this true also in other strains, although body weight and tail-snout lengths are generally less in female mice than in males.

Summary and Conclusions. Osteoarthritis of the knees was less frequent and severe in 16 month old female than male STR/1N mice. Orchiectomy, performed at 4-6 weeks of age, failed to reduce the amount of joint disease in males. Femur length was increased in the castrate animals.

Mrs. Priscilla Auvil and Mr. Fred Bell, Jr. gave capable technical assistance.

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Erythropoietic Activity of Tissue Homogenates.* (27070)

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An erythropoietically active factor has been unequivocally demonstrated in the plasma of a wide variety of mammals, including the human, by many investigators. Early attempts to find a similar factor in tissues, using extraction procedures effective for plasma, were not successful(1). Current tissue work employing other extraction methods and assays of increased sensitivity have been more fruitful. Naets(2) has demonstrated erthropoietic activity in saline extracts of dog kidney, liver and spleen. Lagrue *et. al.*(3) have found similar activity in phosphate buffer extracts of rabbit kidney. Their preparations of spleen, liver and lung were inactive.

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In a preliminary report(4) we indicated that homogenates of muscle, spleen, liver, kidney and brain, prepared from normal and phenylhydrazine anemic rats, were capable of stimulating erythropoiesis. The present communication reports results of extension of these studies.

Materials and methods. Albino rats of a Sprague-Dawley strain were used. Animals serving as tissue donors were retired, female breeders weighing 300 to 350 g. Recipient, experimental animals were females weighing 180 to 220 g. Tissues tested were brain, kidney, liver, muscle and spleen. They were obtained from ether anesthetized donors following aortic exsanquination, and scalpel minced in a petri dish over ice. The mince from 16.0 g of organ was twice washed with 100 ml of modified Tyrode's solution (contained 1% glucose) at 5°C., centrifuged at 3000 r.p.m. in the cold and supernate discarded. The sediment was glass homogenized at 5°C. in a small quantity of Tyrode's solution. Homogenates were prepared fresh daily for injection. In certain instances tissues were frozen in acetone dry ice for future homogenate preparation. Frozen and fresh samples behaved similarly in these experiments. Homogenates were prepared in like fashion from rats rendered anemic with phenylhydrazine. Tissues from these latter animals were obtained when their hematocrits had fallen to between 20 and 25%.

Boiled homogenates, from either normal or anemic donors, were prepared by adjusting the pH of the homogenate to 5.5 with 1 N HCl and boiling for 10 min.

Supernates from homogenates were prepared by centrifugation at 40,000 g for ½ hr in the preparative ultracentrifuge. Concentrated liver supernate was prepared after ultracentrifugation by lyophilization and subsequent dialysis against 1% glucose.

In 2 experiments homogenates were prepared, in a manner similar to that for the rat, from the kidney and liver of normal rhesus monkeys.

All preparations were assayed for erythropoietic activity in dehydrated rats. This is a sensitive assay animal previously described(5), in which has been produced a relative polycythemia with consequent reduction in erythropoietin levels. Water was withheld for 65 hr prior to beginning injection of the test materials. The animals were then injected intraperitoneally daily for 4 days with the materials to be assayed, plus sufficient additional 5% glucose in saline to give 1.5 ml of fluid per 100 g original body weight per day. During the entire period food was allowed *ad libitum*. Homogenates were administered at the rate of 1.0 g (original wet tissue weight) per day. Supernates were calculated to represent 1.0 g original wet tissue weight per day. In the case of concentrated liver supernate, the material was injected in an equivalence of 4.06 g per day. On the fourth day the test animals were given 1.0 μc Fe^{59} I.V. Twenty-four hours later the animals were sacrificed by aortic exsanquination under ether anesthesia. Reticulocyte counts were prepared (dry cresyl-blue method) and incorporation of Fe^{59} into circulating RBC was determined by standard scintillation methods. Control animals received 5% glucose in saline, 1.5 ml/100 g initial body weight per day.

To exclude non-specific effects, groups of test animals were studied following injection of bovine serum albumin (0.2 g/day), gum acacia (25 and 50 mg/day) and methylcellulose (25 and 100 mg/day).

Since RBC retained in the homogenates could conceivably account for erthropoietic activity, quantitative determinations of this residuum were made utilizing Fe^{59} labelled red cells. It was found that a total dose of retained RBC ranging from 0.01 ml to 0.2 ml, depending upon the tissue used, could be expected to be injected into the test animals. Accordingly, groups of assay animals were studied following injection with whole hemolysates of RBC (0.05 and 0.0025 ml/day) and whole, washed RBC (0.05 ml/day).

Results and discussion. The results of the various experiments are summarized in Table I. Tissue homogenates whether from normal or anemic animals produce a consistent and similar erythropoietic response when injected into the rat. This activity is not significantly altered by boiling for 10 minutes at pH 5.5. In centrifuged preparations of normal and ane-

TABLE I. Assay of Erythropoietic Activity.

Material Injected	24 hr Fe^{59} Incorporation in RBC (%)	Reticulocytes (%)	
Brain Homogenate	18.95 ± 7.43*	2.7 ± 1.4*	(11)
Kidney "	15.75 ± 4.87	2.9 ± 1.0	(13)
Liver "	20.83 ± 7.03	2.5 ± 1.5	(15)
Spleen "	20.25 ± 6.65	1.5 ± .6	(3)
Muscle "	17.30 ± 3.43	2.9 ± .8	(8)
Kidney " (Anemic)	21.81 ± 1.62	4.7 ± 2.5	(4)
Liver " "	16.89 ± 6.51	3.2 ± 3.0	(8)
Spleen " "	18.74 ± 10.08	2.3 ± .9	(16)
Liver " (Boiled)	13.56 ± 6.76	1.9 ± 1.5	(7)
Muscle " "	17.30 ± 7.95	3.5 ± 1.7	(4)
Brain " "	18.27 ± 6.99	2.1 ± .5	(3)
Kidney " "	14.22 ± 3.66	2.9 ± 1.3	(4)
Spleen " (Anemic Boiled)	15.78 ± 6.96	1.8 ± .9	(10)
Spleen Supernate	9.12 ± 3.22	.9 ± .7	(11)
Spleen " (Anemic)	12.67 ± 3.23	1.4 ± .4	(11)
Spleen Sediment	22.82 ± 8.94	2.1 ± 1.1	(12)
Spleen " (Anemic)	15.90 ± 5.35	2.0 ± .9	(12)
Liver Supernate (Concent.)	15.66 ± 5.12	2.4 ± .4	(4)
Kidney Homog. (Monkey)	11.66 ± 2.21	1.9 ± .7	(4)
Liver " "	13.28 ± 8.02	2.3 ± 1.2	(4)
Bovine Albumin	6.13 ± 2.29	.4 ± .4	(3)
Gum Acacia (25 mg/day)	3.21 ± 1.07	.3 ± .2	(3)
" " (100 mg/day)	4.82 ± .84	.9 ± .2†	(2)
Methylcellulose (25 mg)	5.59 ± 2.10	1.5 ± .9	(3)
" (100 mg)	5.53 ± 4.55	1.3 ± 1.4	(3)
Hemolyzed RBC (.05 ml)	8.44 ± 2.98†	1.0 ± .3	(4)
" " (.0025 ml)	4.69 ± .93	.7 ± .4	(4)
Whole RBC (.05 ml)	4.09 ± .33	.8 ± .2†	(4)
5% Glucose/Saline (Control)	5.65 ± 2.51	.4 ± .3	(114)

* Mean ± S.D.
Figures in parentheses indicate number of animals in group.
Underlined values statistically significant, P = .01 or less.

† statistically significant at P = .05.

mic spleen, activity is found in both supernate and sediment. Supernates of muscle, kidney and liver (not here tabulated) demonstrated little activity. However, when the liver supernate was concentrated 4-fold significant activity was seen.

That the erythropoietic activity of tissue homogenates is not species specific is demonstrated by the erythropoietic response produced by kidney and liver homogenates from monkeys. That the erythropoietic activity is not a non-specific protein or inert material response is suggested by the failure of bovine albumin and gum acacia to engender erythropoietic responses. Methylcellulose while not increasing Fe^{59} incorporation did produce a reticulocytosis. The mechanism here is obscure. The hemolytic phenomenon produced by methylcellulose in rats normally requires a much longer period of injections before it becomes apparent(6).

Whole hemolysates of red cells in a total dose of 0.2 ml appear capable of stimulating erythropoiesis, though the response is small. Equal quantities of intact red cells have no effect. In the homogenate experiments residual red cells in this quantity are found only in spleen preparations. It appears unlikely that the erythropoietic effect of tissue homogenates can be attributed to their red cell content.

TABLE II. Blood Volumes.

Animals	Blood Vol* % Initial Body Wt	Blood Vol % Final Body Wt	RBC Mass % Initial Body Wt	RBC Mass % Final Body Wt	
Muscle Homogenate	3.37 ± .40	5.09 ± .81	1.95 ± .22	2.94 ± .37	(4)
Control	3.27 ± .18	4.80 ± .00	1.92 ± .07	2.83 ± .21	(3)

* Mean ± S.D.

Figures in parentheses indicate number of animals.

However, the effect of red cell hemolysates is of interest, for it may represent a part of a normal feed-back mechanism controlling erythropoiesis.

It is possible that large quantities of injected protein homogenate could cause a relative reduction in red cell mass by increasing plasma volume. This could stimulate erythropoiesis in an animal relatively polycythemic from dehydration. To test this possibility blood volumes were obtained (using standard Fe^{59} labelling techniques) on control dehydrated animals and dehydrated animals receiving 1.0 g muscle homogenate per day. The results presented in Table II indicate that no blood volume shifts occur, and that this factor cannot account for the erythropoietic effects observed.

Lowering of plasma iron levels with resultant increase in plasma Fe^{59} specific activity could account for the Fe^{59} results observed. Naets has demonstrated that this may occur when dog tissue homogenates are injected into the rats(2). From our own results the increase in specific activity would require a reduction in plasma iron levels to 30% or lower of control. As judged from Naets' data this is an unlikely possibility. In addition our Fe^{59} results, as a measure of erythropoiesis, are corroborated by substantial elevations in reticulocyte counts.

The current findings are contrary to previously recorded results(1,7). The past inability of many investigators, including ourselves, to demonstrate erythropoietic activity in tissues is most probably related to the methods of preparation and utilization of less sensitive assay procedures.

Whether the erythropoietic activity of tissue homogenates is mediated by the same principal (erythropoietin) which has been found in plasma is unknown. In both instances the materials retain activity after boiling. Dialysis of the homogenates against 1% glucose causes no loss of activity. Plasma activity is not lost by dialysis against water. If the plasma and tissue factors are the same, it would appear that the distribution is ubiquitous, and that the kidney, as has been suggested(8), is not the only site of erythropoietin production.

Summary. Homogenates of rat liver, kidney, brain, spleen and muscle prepared in modified Tyrode's solution and injected into the rat are capable of stimulating erythropoiesis as measured by Fe^{59} incorporation into red cells and reticulocyte counts. The activity of the homogenates does not appear to be a nonspecific effect. Certain points of similarity are noted between the homogenates and plasma erythropoietin.

The authors wish to thank Mrs. Stella Karras, Miss Evelyn Rau and Miss Naomi Lupel for excellent technical assistance.

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Enhancement of Cesium^{137} Excretion by Rats Fed Potassium-Supplemented Diets.*‡ (27071)

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Enhancement of radiocesium excretion is of importance to man, as Cs^{137} is a high yield product of uranium and plutonium fission and ranks high on the list of potentially hazardous by-products of peaceful applications of nuclear energy. Published studies regarding enhancement of Cs^{137} excretion through increased potassium intake are still somewhat inconclusive. Mraz *et al.*(1) found that acutely administered Cs^{137} was lost more rapidly from rats when dietary potassium levels were increased. Similar findings were reported for other species(2,3). Ogawa *et al.*(4) found that ingested potassium chloride had little, if any, effect on retention of acutely administered Cs^{137}. Wasserman and Comar(5) found no beneficial effect of increased dietary potassium on retention of chronically administered Cs^{137} by rats.

Some experimental results are questionable because potassium-deficient semisynthetic diets were fed to control animals. Feeding of diets deficient in a specific element is known to cause pronounced deviations from the metabolic pattern normally obtained for that element. In addition, potassium-deficient diets are often deficient in other elements, such as magnesium, and can cause renal damage(6,7). Results of this study, obtained by whole body counting techniques, represent another contribution towards the solution of this problem.

Materials and methods. Thirty male Sprague-Dawley rats, 84 days old (average weight, 317.5 g), were divided into 5 equal groups. One group was fed ground Purina Lab Chow (basal diet); another was fed a commercially supplied potassium-deficient diet; and the remaining 3 groups were fed basal diet supplemented with varying amounts of potassium added as chloride. Potassium concentration was about 1% for the basal diet (8), and 5, 8, and 11% for the supplemented diets. A Patterson-Kelley twin-shell laboratory blender was used to facilitate mixture of basal diet with the potassium supplements. All animals were fed basal diet for about 1 week for acclimation to pulverized food and special feeders.

Immediately after intraperitoneal injection of 0.65 μc carrier-free Cs^{137} per animal, each group was placed on its specific dietary regimen. Animals were radioassayed individually at 30 minutes following injection and at suitable intervals therafter in a 4π whole body liquid scintillation counter(9). Biological retention of Cs^{137} at each time of measurement was calculated by the following expression: Biological Retention = (Animal Count Rate/Standard Count Rate)$_t$/(Animal Count Rate/Standard Count Rate)$_o$ in which subscripts t and o refer to time after Cs^{137} injection. This expression corrects for minor day-to-day fluctuations in the counting system.

Approximately 80 days after injection, the 3 surviving animals maintained on the potassium-deficient diet were placed on basal diet for the remainder of experiment.

Results and Discussion. Mean whole body retention values as a function of time are given for each group in Fig. 1. The time-course of Cs^{137} retention for control animals is similar

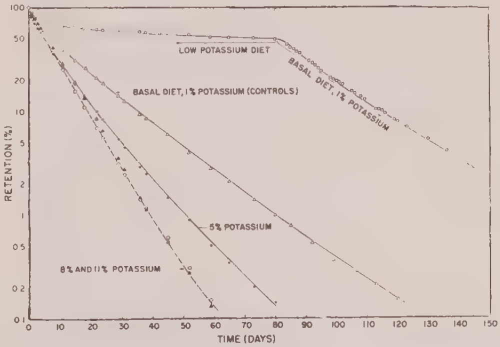

FIG. 1. Effect of dietary potassium on retention of Cs^{137} administered intraperitoneally to rats.

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‡ Appreciation is expressed to G. A. Trafton and B. M. Cummins for technical assistance.

TABLE I. Parameters of Cesium^{137} Retention Functions for Normal Male Sprague-Dawley Rats.

	No. of Rats	Component	a* (%)	k* (fraction/day)	T_b† (days)
This Study	6	1	17.26	.84110	.82
		2	28.15	.10869	6.38
		3	54.47	.05014	13.82
Previous Study(10)	6	1	17.34	.75918	.91
		2	32.38	.10094	6.87
		3	50.19	.05181	13.38

* From $R_t = \sum^{n} (a_i e^{-k_i t})$, where R_t is retention at any day t, and a_i and k_i are intercept and rate constants, respectively.

† $T_b = \frac{0.693}{k}$

to that previously observed for normal rats at this Laboratory(10).

Table I compares retention parameters obtained for control animals in this experiment with those previously obtained for normal rats. In both cases, retention parameters were obtained by computer analyses based on iterative least square calculations. Details of this curve-fitting procedure have appeared elsewhere(10). Values in Table I indicate how precisely the time-course of retention can be measured and described by whole body counting and computer curve-fitting techniques. Further, by integrating the retention functions (Table I) between o and ∞ time, we find the total areas agree to within ±2%.

It is apparent from Fig. 1 that the potassium-deficient diet caused increased retention of Cs^{137} and, therefore, does not serve as an adequate base line or control for studies designed to assess effects of dietary potassium on Cs^{137} excretion. It is also apparent that when these animals were returned to the basal diet, the retention pattern approximated that of control animals on an adequate potassium diet.

All groups receiving supplementary dietary potassium lost Cs^{137} more rapidly than the control group. Increased Cs^{137} excretion was not linearly related to potassium content of the diet, as no further increase was observed when dietary potassium was increased above 8%.

Fig. 1 shows that the initial Cs^{137} burden of animals on the 5% potassium diet decreased by a factor of 100 in 50 days, whereas the time required for a similar reduction in the initial body burden of animals fed basal diet containing 1% potassium was 80 days. At 20 days after injection, the Cs^{137} burden of animals maintained on the 5% potassium diet was half that of animals fed basal diet. This experiment simulates conditions of accidental Cs^{137} contamination and suggests the order of effect that may be expected from immediate therapeutic measures based on increased dietary potassium. In view of possible practical applications of such therapy, similar experiments will be repeated on larger mammalian species.

Summary. Normal rat diet supplemented with potassium at concentrations of 5, 8, or 11% increased the excretion of a single intraperitioneal dose of Cs^{137} from rats as compared with control rats ingesting normal rat diet without added potassium. Twenty days after injection, the Cs^{137} burden of rats maintained on 5% potassium diet was half that of animals fed basal diet. Animals fed a semisynthetic low potassium diet retained Cs^{137} more tenaciously than animals on the normal diet.

Increasing dietary potassium intake simultaneously with exposure to Cs^{137} does have the beneficial effect of enhancing Cs^{137} excretion in the rat.

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Production of Interferon by Suspensions of Human Leucocytes.[†][‡] (27072)

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Multiplication of several animal viruses in suspensions of leucocytes has been previously demonstrated(1). Since it is now well established that cultures of human cells produce interferon-like substances we considered it of interest to determine whether human leucocytes of the circulating blood exposed to virus might respond in the same manner. The results of such experiments form the basis of this preliminary communication.

Methods and materials. Suspensions of Leucocytes. Procedures for obtaining venous blood and preparation of suspensions of leucocytes utilizing Bacto-Phytohemagglutinin (Difco) have been previously described(1). After cell counts, washed leucocytes (500,000-5 million/ml) were resuspended in 5-10 ml of medium consisting of 5% heat inactivated horse serum, 5% beef embryo extract, 45% bovine amniotic fluid and 45% Hanks' balanced salt solution. Leucocyte preparations were incubated in rubber stoppered[§] prescription bottles 10 cm × 4 cm kept on stationary racks at 37°C. Virus was in most instances added one hour after preparation of cells.

Viruses. Relevant data on viruses employed are as follows: Sendai virus (F. Davenport) allantois of embryonated egg, 27 passages; Sindbis virus (R. Taylor AR-339) human amnion (HA) cells, 10 passages; Polio virus II (MEF_1) chick embryo cell cultures 143, HA cells, 2 passages; Polio virus I (Brunhilde) human uterus cell cultures, 3 passages; Measles virus, Edmonston strain, human kidney cells, 29 passages.

Preparation of Stock Sendai Virus. Sendai virus was grown in 9-11 day embryonated eggs. The inoculum, consisting of a 10^{-3} dilution of stock virus was introduced into the allantoic cavity. After 72 hours incubation at 35°-37°C, allantoic fluid was harvested, centrifuged for 10 minutes at 500 rpm and the supernate centrifuged at 4°C for 2 hours at 110,000 g (Spinco L preparative centrifuge #40 rotor). The sediment was resuspended in a mixture of 50% bovine amniotic fluid medium and 50% skimmed milk and stored at −70°C.

Virus Assay. Infectivity titers were calculated by the formula of Reed and Muench. Sendai virus was titrated in embryonated eggs (EID_{50}) taking as criterion of infection appearance of hemagglutinin in allantoic fluids. Sindbis, poliovirus and measles viruses were prepared and titrated in HA cells. In this system more than 25% cell destruction was considered evidence of viral multiplication. Preparation and maintenance of HA cells has been previously described(2).

Antiserum. Anti-Sendai rabbit serum (HAI-titer = 1:1280) was obtained through the courtesy of Dr. J. Lewis of the Sterling Winthrop Co., Rensselaer, N. Y.

Assay of Interferon. All preparations to be tested for interferon were centrifuged at 110,000 g at 4°C for 2 hours. The supernate (interferon) was shown to be free of infectious virus and hemagglutinin and as an additional precaution to eliminate infectious virus, anti-Sendai rabbit serum in excess was added in certain instances. Interferon was assayed in primary tube cultures of HA cells previously incubated in stationary racks at 35°-37°C for 4 or more weeks. Two fold dilutions of interferon (2-3 tubes/dilution) were incubated with cells for 18-24 hours prior to inoculation of 100 $TCID_{50}$ of Sindbis virus. Complete destruction of cells in control cultures regularly occurred in 3 days. Interferon titer was expressed as the highest dilution

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[†] The author gratefully acknowledges the constant help and guidance of Dr. J. F. Enders. We also wish to express our gratitude to Miss Alison Downie for technical assistance, Dr. E. Engel for technical advice, Dr. S. V. Kevy and Dr. H. McDevitt for arranging to supply us with blood specimens.

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[§] The West Co., Phoenixville, Pa.

which protected more than 75% of the cell sheet in at least one of 2 cultures for at least 3 days after complete destruction of cells had occurred in control cultures.

Experimental. Production of Interferon by Leucocytes. Previous experiments suggested that after inoculation of high concentrations of chick-adapted Sendai virus into primary cultures of human amnion cells, an incomplete cycle of viral replication ensued. This was associated with rapid production of high titers of an interferon-like substance(3). Sendai virus was therfore chosen as the principal interferon-inducing virus and was inoculated in high concentrations (10^7-10^8 EID_{50}/ml) into suspensions of leucocytes ranging from 500,000-5 million cells/ml. On serial passage of this chick adapted virus in human leucocytes no multiplication of infecious virus was demonstrated. Thus after inoculation of $10^{7.8}$ EID_{50}/ml of virus, only $10^{4.7}$ EID_{50}/ml was recovered after 72 hours. No infectious virus was detected in the medium after 2 further passages of this material utilizing fresh preparations of washed leucocytes. On two occasions measles virus (Edmonston strain) was employed as the test agent for interferon production since it had been previously demonstrated that this strain multipled in suspensions of human leucocytes(1).

Examination of stained preparations of leucocytes 3 and 7 days after addition of virus revealed that the great majority of remaining cells were mononuclear. Only a few polymorphonuclear cells were observed. No morphologic cellular abnormalities were noted though tests for viability were not performed.

Leucocyte suspensions prepared from the peripheral blood of 20 healthy and ill donors were tested for interferon production (Table I). After inoculation of the suspensions with either Sendai or measles virus, interferon in varying titers was detected in 21 of 22 preparations (15/16 patients). In one case (pt. #16) no interferon was demonstrated. The leucocytes from this person were inadvertently maintained in a medium subsequently shown to be markedly toxic for primary cultures of human amnion cells. It is probable therefore that this medium was toxic for leucocytes as well, though this was not established.

In no instance was interferon found in uninoculated suspensions: (10 suspensions representing 10 subjects). In 2 patients (pts. #18 and #19) with typical measles attempts to isolate the virus from whole blood were unsuccessful, as well as an attempt to isolate this virus from a suspension of one patient's leucocytes which was incubated for 3 days at 37°C.

In considering the results presented in Table 1, it should be emphasized that because of a number of variables, interferon titers cannot be accurately compared. The number of leucocytes in different suspensions varied between ½ and 5 million cells/ml and different lots of cultures of human amnion cells were employed in assaying interferon. It is possible that the sensitivity of primary cultures of amnion cells varies in respect to both interferon and challenge virus.

Identification of Inhibitor. Though all interferon preparations were ultracentrifuged to remove infectious Sendai virus, only representative preparations were subjected to various physical and chemical tests. It was found that neither ultracentrifugation nor antiserum to the interferon-inducing virus diminished the viral inhibitory effect of these preparations. Dialysis for 24 hours at a pH of 4, and heating at 56°C did not affect the interferon titer; but incubation with crystalline trypsin (1 mg/1 ml 37°C 1 hour) reduced the titer from 1:384 (pt. #2) to <1:8.

Quantitative Relationship between Number of Leucocytes and Yield of Interferon. From Table II it is apparent that after inoculation of 10^8 EID_{50}/ml of chick adapted Sendai virus into suspensions of leucocytes decreasing by a factor of 10 the concentration of interferon found 72 hours later was roughly proportional to the number of cells. Though erythrocytes were present in all leucocyte preparations they played no role in interferon production since most of them were lysed shortly after addition of Sendai virus. This agent has been shown to cause hemolysis(4). Moreover, no interferon was detected after exposure to the virus in suspensions of erythrocytes which contained very few leucocytes (Table II).

Rapid Production of Interferon. After infection of primary cultures of human amnion cells with Sendai virus interferon was demonstrated in high titer within 12 hours after

TABLE I. Production of Interferon by Human Leucocytes after Exposure to Virus.

	Patient	Age	Sex	Disease	Interferon-Inducing Virus	Interferon Titer[1] (hr of harvest)[2]	
1.	EE	35	M	Normal	Sendai	24	(48 hr)
2.	IG	32	M	"	"	384[3]	
				"	Measles	>96	
				"	Polio I	<4	
	IG	32	M	"	None	<4	
				"	Sendai	<4	(2 hr)
				"	"	256	
	IG	32	M	"	"	>384	
3.	BJ	45	F	Acute Barbiturate Ingestion	"	24	
4.	CF	80	F	Myocardial Infarct Convalescent	"	>192[4]	
5.	PT	78	F	Congestive Heart Failure	"	>3072	
6.	AP	80	F	Cirrhosis	"	1024	
7.	CG	28	F	Obesity	"	>768	
	CG	28	F	"	"	64[5]	
8.	JY	3	F	Acute Myelomonocytoid Leukemia[6]	None	<4	
					Sendai	>192	
9.	JJ	82	M	Chronic Lymphatic Leukemia[7]	None	<4	
					Sendai	24	
10.	PT	2	M	Acute Myelomonocytoid Leukemia with Acute Varicella (1 day)[8]	None	<4	
					Sendai	96	
11.	ML	2	F	Acute Lymphoblastic Leukemia[9]	"	64	
12.	FE	16	M	Congenital Agammaglobulinemia	"	32	
					Measles	12	
					Sendai	>1024	
13.	JW	18	M	idem.	"	32	
14.	EA	11	M	"	"	>256	
15.	DW	14	M	Aseptic Meningitis	None	<4	
					Sendai	>384	
16.	DG	3	M	" "	None	<4	
					Sendai	<4	
17.	NM	14	M	Mumps Sx. 60 hr duration	None	<4	(3 & 7 days)
18.	IN	4	M	Measles 24 hr rash	"	<4	" " " "
19.	JH	2	F	? Measles 24 hr rash	"	<4	" " " "
20.	HB	2	M	? Infectious Mononucleosis-5 days duration	"	<4	" " " "

[1] Expressed as reciprocal of dilution of medium that inhibited 100 $TCID_{50}$ of Sindbis virus.

[2] All media assayed for interferon were collected 72 hours after viral inoculation unless otherwise specified.

[3] Interferon titer of this preparation was >192 against Poliovirus II (MEF_1).

[4] Interferon titer <4 when leucocytes were subjected to 2 cycles of freezing and thawing prior to inoculation of Sendai virus.

[5] Leucocytes were incubated for 3 days prior to inoculation of challenge virus, to decrease number of polymorphonuclear leucocytes in suspension.

[6] WBC count in peripheral blood 245,000/mm^3. On examination of stained preparations of peripheral blood all cells were believed to be "blasts."

[7] WBC count in peripheral blood 10,000/mm^3 75% lymphocytes.

[8] WBC count in peripheral blood 6,500/mm^3 60% "blasts."

[9] WBC count in peripheral blood 550/mm^3. All cells seen on examination of stained preparation either immature "blasts" or lymphocytes.

TABLE II. Relationship Between Number of Leucocytes and Production of Interferon.

No. of Leucocytes/ml Suspension	Interferon Titer*
5,200,000	>3072
520,000	384
52,000	32
5,200	12
520	<2
52	<2
5	<2
Erythrocytes Suspension†	<2

* Reciprocal of dilution of medium, 72 hr after addition of virus.

† Containing <4000 WBC/ml.

infection and maximal titers were attained at 24 hours(3). Though medium from leucocyte suspensions was routinely harvested for interferon assay 72 hours after inoculation, it can be seen from Table III that interferon was liberated in high titer in one experiment as soon as 6 hours after inoculation.

Production of Interferon by Cells of Patients with Agammaglobulinemia. Interferon was formed as readily by leucocytes taken from 3 patients with congenital agammaglobulinemia as it was by cells from normal subjects (Table 1). The clinical course of patient #12 F.E. has been previously described(5,6). He had not received gamma globulin for over one year and his gamma globulin level was usually 25 mg% (normal 600-800 mg%). Patient #13 J. W. is the uncle of Patient #14 E. A.(7). Gamma globulin was administered to uncle and nephew one month prior to testing of their leucocytes and gamma globulin levels on the day leucocytes were withdrawn were 40 mg% and 250 mg% respectively.

Attempts to Detect the Presence of Virus by Assaying Media for Interferon. It is well known that viremia occurs in certain viral diseases. It seemed possible therefore that after incubation of leucocytes taken during the viremic phase, interferon might be produced by leucocytes infected *in vivo*. Emergence of interferon in the medium would provide a criterion for the presence of virus. Attempts were therefore made to detect interferon in the media of uninoculated leucocytes from a normal subject (#2), 3 leukemic patients (Nos. 8,9,10) and 6 patients suffering from acute viral diseases (Nos. 15,16,17,18,19,20) (Table 1). No interferon was demonstrated.

Despite these negative results, an attempt was made to demonstrate presence of the virus of rubella by adding various materials from patients with this infection to suspensions of leucocytes and subsequently assaying the medium for interferon. To preparations of washed leucocytes were added aliquots of throat washings and blood from 3 patients with typical rubella. Materials from 2 of these patients had been collected one day before the onset of rash as well as on the day of rash and had been stored at −70°C for one year prior to testing. Material from the 3rd patient was collected on the day prior to onset of rash and had been stored at −20°C for 7 weeks. Two passages were made in leucocyte suspensions (4 million/ml). No interferon was detected in the medium after assay on the 3rd and 7th day of either the 1st or 2nd passages.

Discussion. A consideration of the various properties of the viral inhibitory factor produced by human leucocytes after infection with Sendai and measles virus would seem to justify employing the term interferon(8). That this interferon was formed and liberated by the leucocytes themselves was supported by the following facts: 1) no interferon was demonstrated in the original viral inoculum (Sendai) which had been prepared by ultracentrifugation of infected allantoic fluid and resuspension of the pellet in medium free of interferon. 2) no interferon was demonstrated in medium nourishing uninoculated leucocytes. 3) no interferon was present in the medium nourishing leucocytes ½-2 hours after inoculation of virus (Table 1, pt. #2 and Table III). 4) amount of interferon released appeared roughly proportional to number of leucocytes. 5) no interferon was detected when leucocytes were lysed by rapid freezing and thawing prior to introduction of virus (patient #4, footnote 4). 6) no interferon was detected in a suspension of erythrocytes exposed to virus.

Since interferon was produced shortly after inoculation there can be little doubt that surviving cells originally present in the donor's blood were responsible for production of interferon rather than descendants of these cells. Evidence was obtained that mononuclear cells were capable of producing interferon. Thus leucocytes incubated for 3 days at 37°C prior to inoculation (case #7, footnote 5) produced interferon. Very few polymorphonuclear cells were seen on examination of stained preparations of these cells at time of inoculation. Moreover, only lymphoblasts or lymphocytes were observed in a differential count of leucocytes of patient #11, yet interferon was

TABLE III. Production of Interferon by Leucocytes at Various Times After Exposure to Virus.

Time in hr	½ hr	1 hr	6 hr	12 hr	16 hr	24 hr	40 hr
Pt. #5 2,600,000 cells/ml	—	<4	—	—	1024	—	1024
Pt. #6 3,700,000 cells/ml	<4	—	>768	768	—	1024	—

— indicates not tested.

elaborated by these cells. Whether or not polymorphonuclear cells also produce interferon cannot be determined from the data now available.

The number of variables in the experimental system precluded quantitative comparison of interferon titers. It should be entirely feasible, however, to control these variables and thus derive significant information on rate and degree of interferon production by leucocytes from patients with a variety of diseases.

Whether interferon-like substances are actually produced by leucocytes *in vivo* remains uncertain though there appears to be no reason to consider this unlikely. In this regard it is of interest that leucocytes from patients with classical congenital agammaglobulinemia produce interferon *in vitro*. It is well known that this group encounters relatively little difficulty with naturally acquired viral diseases and it has been suspected that their recovery may depend on factors other than conventional antibody.

Though we have been unable to demonstrate production of interferon by uninoculated leucocytes taken from patients with acute viral diseases, it should be emphasized that in these cases no virus was isolated from peripheral blood. If leucocytes were taken during the viremic phase of a disease such as measles it is entirely possible that interferon might be produced by these cells when subsequently maintained *in vitro*. This supposition would seem to find support from the following considerations: 1) measles virus has been isolated from the buffy coat of leucocytes from patients with measles(9). 2) measles virus has been propagated in suspensions of human and monkey leucocytes(1). 3) measles virus, as we have shown on 2 occasions, does induce production of interferon by leucocytes *in vitro*.

We were unable to detect interferon in the medium of normal leucocytes inoculated with material from patients with rubella. However, this approach to the detection of certain non-cytopathogenic agents might prove to be of considerable value, since it is possible that such agents adapted to the human host might multiply in human leucocytes maintained *in vitro* and reveal their presence by the production of interferon.

Summary. Suspensions of leucocytes from 2 normal donors and 18 patients with various diseases, including 3 patients with congenital agammaglobulinemia were exposed to Sendai or measles virus and maintained *in vitro* for several days. Production of interferon occurred in 21 of 22 preparations. It is suggested that interferon-like substances may be liberated by leucocytes *in vivo*, after infection with some viruses and that production of interferon by leucocytes may provide a criterion for presence of non-cytopathic viruses.

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Fetal Plasma Potassium.* (27073)

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Christianson and Jones(1) have summarized evidence that fetal plasma potassium concentration is far higher than the normal adult range. Reported values are as high as 17 meq/l(2), while others are around 9 meq/l,

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Since these levels are in the lethal zone for the adult, and since some of our analyses and those of others(3-6) were far below this level, our data were analyzed for the cause of the variation. The elevated K concentrations appear to be associated with conditions of fetal hypoxia, and there is no evidence in our series that fetuses normally exist *in utero* with such high concentrations.

Procedure. On days 19-21 of gestation, blood of fetal rats has been collected in hematocrit capillaries from the punctured chin sinus and centrifuged immediately. Hemolysis was not present. In some experiments, the samples were collected in less than one minute after delivery by Caesarean section. In others, the whole uterus had been removed, the fetuses delivered from their membranes in less than 14 minutes, and the blood sampled in less than 30 minutes. In still others, the dissections required 20-40 minutes, with sampling completed before the end of an hour. Some fetuses survived each procedure. Potassium was determined by Beckman B flame photometry.

Results. Table 1 summarizes the evidence that fetal plasma K is elevated in proportion to the duration of conditions resulting in hypoxia. When fetuses were sampled before hypoxia could become severe, plasma K concentration was in the upper half of the range for normal adults. Only in moribund or dead fetuses were concentrations of 9 meq/l and above observed. In littermates that were able to establish rhythmic respiration, concentration seldom exceeded 7.5 meq/l.

Discussion. The discrepancies between values in the literature may be explained similarly, as also indicated by Brandt, Harned, and Cooke(4), who have reported fetal values in the normal adult K range for normal fetuses, followed by significant increases after hypoxia was induced. Samples were obtained through indwelling catheters. Other studies in which the fetus was in good viable condition report fetal plasma K concentrations in the normal adult range for several species(4,5,6). All of these laboratories have been concerned with fetal respiratory problems as well as electrolytes and are perhaps more aware of the necessity for obtaining samples before hypoxia develops. Others apparently have been less cautious in avoiding sampling under conditions in which hypoxia was probable, e.g., tissues brought from the slaughterhouse or drainage of blood after decapitation. High values comparable to our delayed dissections are reported in these studies.

It is well known that plasma K rises rapidly as death approaches in adults, and there is reason to believe that fetal plasma K behaves similarly. Battaglia, *et al.*(7) have shown that fetal osmotic pressure rises rapidly on brief exposure to hypoxia. At least a part of the increase is undoubtedly due to increased K. Whether the K elevation is quantitatively different from that of adults is uncertain. Nevertheless, plasma K concentrations of fetuses measured after hypoxia of only a few minutes' duration should not be designated "normal".

Conclusion. Fetal plasma potassium concentration is normally within the range reported for adults. Markedly elevated K concentrations are observed after brief periods of hypoxia. Concentrations up to 17 meq/l may be anticipated if the fetus dies.

TABLE I. The Effect of Hypoxia on Fetal Plasma Potassium.

Duration of stay within membranes after removal of uterus from mother, min.	Respiratory responsiveness at delivery	Mean plasma K, meq/l ± S.D.	Maximum plasma K, meq/l	Number of fetuses	Number of litters
<1	Good	5.6 ± 0.6	6.4	13	3
<14	Present or rhythmic	6.2 ± 1.1	7.5	17	5
	Absent	9.3 ± 1.3	12.1	7	7
	All fetuses	7.1 ± 1.8	—	24	8
20-40	Present but not rhythmic	9.8 ± 1.2	12.6	11	5
	Absent	12.8 ± 2.1	16.6	31	9
	All fetuses	12.0 ± 2.3	—	42	13

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Western Encephalitis Virus in Massachusetts.*† (27074)

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(Introduced by Geoffrey Edsall)
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This report describes the first recorded recovery of western encephalitis (WE) virus from mosquitoes and birds in Massachusetts and serological evidence indicating that a considerable amount of enzootic activity of the virus has occurred there.

The initial isolation of WE virus east of the Appalachian Mountains was obtained from an immature house sparrow *(Passer domesticus domesticus)* collected in New Jersey during Sept. 1953(1). In 1952 the virus was isolated in Louisiana(2) from a loggerhead shrike *(Lanius ludovicianus)*, a Carolina chickadee *(Parus carolinensis)*, a cardinal *(Richmondea cardinalis)*, and from 2 mosquito species *(Culiseta melanura* and *Aedes infirmatus)*. Subsequent isolations of WE virus from mosquitoes, *Culex pipiens* in North Carolina and *C. melanura* in New Jersey, during 1955 and 1956 led to the hypothesis that "——— *C. melanura* may be an endemic vector of WE in the eastern part of the country"(3).

Materials and Methods. In southeastern Massachusetts, mosquitoes were collected alive in New Jersey-type light traps and bird-baited traps, anesthetized with chloroform and identified, grouped by species in lots numbering up to 50, and stored in an electric freezer at −65°C in corked Wassermann tubes until tested. Samples of avian blood were obtained by cardiac or wing vein puncture, diluted 1:5 in a physiological saline solution containing 0.01% heparin, and placed on wet ice for transport to the virus laboratory for testing. Pheasant brain specimens were obtained through the courtesy of Dr. George P. Faddoul‡. Chick embryo tissue cultures(4) were used in testing the mosquitoes and avian specimens for virus.

Suspensions of the mosquitoes were prepared for inoculation into the tissue cultures. To prepare the suspensions, a few drops of diluent were added to each pool of mosquitoes in its Wassermann storage tube. The diluent used was Hanks' balanced salt solution buffered at pH 7.8 with .04 M hydroxymethyl aminomethane, 0.3% bovine albumin Fraction V, and 2% stock antibiotics (5,000 units mycostatin and 10,000 μg each of penicillin and streptomycin per ml sterile distilled water). After this addition, the mosquitoes in the tube were ground with a sterile wood applicator, and enough diluent was added to bring the volume to 2 ml.

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The suspensions were centrifuged 20 minutes in a refrigerated centrifuge at 4000 RPM; Seitz filtration with a Boerner centrifugal filter was occasionally needed to remove bacteria or fungus contaminants. A tissue culture was inoculated with 0.1 ml of the supernatant fluid from each suspension, and the remaining portions were stored at −65°C. Each day for 3 days the tissue cultures were examined for cytopathogenic effect (CPE), and when this was observed a subculture was made. On the third day, all cultures that had shown no change were "blind passed," and these were regarded as negative if no CPE appeared by the end of 3 more days.

The pheasant brain material suspension was tested for virus in chick embryo tissue culture after being ground with alundum in a mortar and pestle.

Transmissible agents isolated in the tissue cultures were identified by complement-fixation techniques on the tissue culture supernatant. If anticomplementary or cross reactions occurred, a chick embryo passage was made and the allantoic fluid was tested by complement-fixation. Eight antibody units of eastern encephalitis (EE)-immune and WE-immune serum were used in the tests, and controls served to exclude cross reactions and nonspecific fixation of complement.

Tests of the avian sera for presence of WE and EE antibody were made by the hemagglutination inhibition (HI) technique of Clarke and Casals(5) and using antigens consisting of crude tissue culture supernatants, together with pyrophosphate buffer. Neutralizing antibody tests by a plaque reduction method previously described(6) were done on selected sera for confirmation.

Results. WE virus was isolated from 26 pools of *Culiseta melanura* and from one pool of *Aedes canadensis* collected during the period August 31-Oct. 6, 1959 in southeastern Massachusetts. Of these positives, 3 were collected at Middleboro and Lakeville, and the remainder were obtained from 2 locations in Raynham, Mass.

Strong complement-fixing (CF) antigen in allantoic fluid was demonstrable by specific hyperimmune sera under controlled conditions for all the WE isolates obtained from mosquitoes.

Unengorged female *C. melanura*, without visible ingested blood in the abdomen, comprised 15 of the pools which were positive for WE virus. The number of specimens in these positive pools ranged from 10 to 50. Three of these pools were collected in light traps, and the other 12 were collected in bird-baited traps.

Engorged female mosquitoes, with recently ingested blood in the abdomen, comprised the remaining 11 positive pools of *C. melanura*. The number of specimens in these positive pools ranged from 15 to 50. All of the positive pools of engorged mosquitoes, including the positive pool of 33 engorged *A. canadensis*, were collected in bird-baited traps.

WE virus was isolated from the brain of a 3-year-old Impeyan pheasant stricken during the summer of 1959 at a wild-animal farm in Middleboro, and the initial isolation was confirmed by reisolation of the virus from a suspension of the original brain material. Tissue culture supernate contained a weak (2+) CF antigen which reacted with WE hyperimmune guinea pig serum but not with EE hyperimmune guinea pig serum. Allantoic fluid passage material contained a strong (4+) CF antigen. Tissue culture hemagglutinin behaved like WE, since it was inhibited by WE convalescent rabbit serum and not by EE convalescent rabbit serum, and its maximum hemagglutination titer was at pH 6.0. One of two 1-week-old chicks inoculated in the breast muscle with tissue-culture-passage material survived and produced WE antibody. Blood collected 12 days after inoculation neutralized stock WE virus, Fleming strain, by reducing the average number of plaque forming units from 120 to 4. An uninoculated control chick bled at the same time did not have WE antibody. Thus, the isolate from the pheasant brain was proved identical to WE virus by reciprocal serological tests.

TABLE I. Hemagglutination Inhibition Antibody Among Massachusetts Domestic Birds Hatched During 1959.

Species	Age in Mo	No. Tested		No. Positive			% Positive		
		WE	EE	WE	EE	Both	WE	EE	Both
Chicken	4-6	181	231	5	15	1	3	6	.5
Turkey	4-6	202	202	54	13	7	27	6	3
Pheasant	4-9	46	96	0	0	0	0	0	0
Totals		429	529	59	28	8	14	5	2

WE virus also was isolated from the blood obtained from a 3-week-old chicken from Bridgewater, Mass. on Aug. 17, 1959, and from 3 pools of unengorged *C. melanura* collected from Raynham, Mass. during Aug. 1960.[§]

In HI tests on 529 domestic birds hatched during 1959, 5 chickens and 54 turkeys were positive for WE antibody; 15 chickens and 13 turkeys were positive for EE antibody, but no antibody was detected among the 96 pheasants tested (Table I). A nonspecific or cross-reaction occurred between WE and EE virus in one of the chicken sera and in 7 of the turkey sera tested. It is not known whether or not other group A viruses occur in Massachusetts; if so, they may contribute to the broad reactivity expected with HI(5).

Discussion. The described isolations of WE virus from mosquitoes, a chick, and a pheasant are the first clear-cut demonstrations of active infection with the virus in Massachusetts. Serologic evidence obtained in 1953 that the virus was present then was questioned(7), but subsequent results (unpublished data) of neutralization tests by the plaque reduction technique(6) on sera obtained from chickens and turkeys in Massachusetts during 1958, 1959, and 1960 substantiated the earlier findings.

The 15 isolations of WE virus from pools of unengorged *C. melanura* constitute the majority of isolations reported from the area east of the Appalachian mountains.

The number of mosquito pools is too small for conclusive statistical determination of the probability that blood from birds used as bait on given nights might have been the source of virus in the 11 positive pools of engorged *C. melanura*. However, there was no difference (chi-square, 5% probability level) between the total numbers of positive and negative pools of unfed and engorged specimens. Since the distribution was random, it would seem that the birds were not a source of virus.

The mosquitoes and the birds from which WE virus was isolated were obtained from the adjoining towns of Bridgewater, Lakeville, Middleboro, and Raynham. The high WE antibody rate obtained in turkeys was due primarily to detection of 34 positive birds among 39 tested, and to 19 positive among another 50 tested, at farms located in East Weymouth and Middleboro, respectively.

The virus of WE is responsible for the majority of human and equine cases of arthropod-borne encephalitis in North America, but it has not been associated with known human disease along the eastern seaboard. The unimportance of WE as a disease-producing agent in man and horse in the eastern United States was suggested as being due to the absence there of a vector as efficient as *Culex tarsalis* is in the West(8). Evidence is accumulating, however, that *Culiseta melanura* is an enzootic vector of WE as well as EE in the eastern United States. In laboratory studies, several eastern mosquito species (e.g., *Aedes triseriatus, A. sollicitans,* and *A. vexans*) have been found to be potential vectors of WE virus(9).

Three elements (virus, vectors, and susceptible hosts) essential for a WE outbreak now have been reported in Massachusetts, New Jersey, and Louisiana. Therefore, it is important to realize the existence of the potential for an outbreak of WE in the eastern United States.

Summary. The first recognized active infections of western encephalitis (WE) in Massachusetts are described. The virus was isolated from 15 pools of unengorged and 11 pools of engorged *Culiseta melanura* and from 1

[§] Virus isolated in 2-week-old mice and chick embryos at Encephalitis Section Virus Lab., Communicable Disease Center, Greeley, Colo.

pool of engorged *Aedes canadensis* collected in southeastern Massachusetts during 1959, and from 3 pools of unengorged *C. melanura* collected in 1960. The virus also was isolated from the brain of an infected 3-year-old Impeyan pheasant and from the blood of a 3-week-old chicken in 1959. WE and EE (eastern encephalitis) antibodies were detected in domestic fowl hatched and reared in widely separated areas in eastern Massachusetts during 1959. Although no human or equine outbreaks of WE have occurred in the eastern United States, the potential for such an outbreak apparently exists.

Addendum. Since this paper was submitted for publication, there have been three isolations of western virus from 1961 collections—two from pools of *C. melanura* and one from the blood of a catbird during the nesting season.

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Effect of Local X-Irradiation on Growth Capacity of Mouse Kidney.* (27075)

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It is well known that cells may continue to enlarge after dosages of X-rays sufficient to block cell division. Renal compensation after unilateral nephrectomy is generally attributed to hypertrophy(1). This system, therefore, lends itself to the study of radiation effects on a growth process characterized mainly by enlargement of existing cells(2).

In the present investigation the following experiments were performed: 1) effect of uninephrectomy on size and DNA content of the remaining kidney in mice of various ages, 2) effect of immediate or delayed local X-irradiation on renal compensatory response. We have confirmed the fact that mitosis plays a relatively minor role in the kidney enlargement following uninephrectomy in the young adult mouse. We have also found this compensatory phenomenon to be radioresistant.

Materials and Methods. CF no. 1 female mice of various age groups received sodium pentothal intravenously and the right kidney was exposed by a dorsal surgical incision, ligated and removed. Care was taken to maintain the integrity of the adrenal gland. Unilaterally nephrectomized mice were sacrificed 5 to 6 days post-operatively and the kidneys taken for DNA analysis by the spectrophotometric method of Schneider(3) as modified by Barton(4). These were compared with the normal kidneys removed from the same animals at time of operation. The values reported for the 2-month-old animals are based on individual microdeterminations; those of mice in other age groups represent pooled samples of several kidneys. In the radiation studies, the left kidney was freed from the adrenal and ovary and exteriorized after removal of the right kidney. Local irradiation of the organ was accomplished through a one centi-

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meter cone, using a GE Maximar 100 source. The radiation factors were: 100 kv, 5 ma, target distance 15 cm, 2014 r/min using 0.1 mm of Al shielding. By adjusting the X-ray cone and the mouse it was possible to irradiate the exteriorized kidney alone without shielding the rest of the animal. Precautions were taken to prevent pressure upon the kidney or its blood supply during irradiation; the color of the exteriorized organ remained normal throughout the exposure. After irradiation the kidney was replaced in the body cavity and the muscle and skin closed separately with interrupted silk sutures.

Results and Discussion. Removal of the right kidney in the normal mouse results in a 50% increase in the wet weight of the left kidney 26 to 38 days post-operatively. The weight of the remaining kidney at this time corresponds to 75-80% of the initial combined weight of both kidneys. The greater part of this increase (40% increase above initial weight, 70% of initial combined weight) is attained within 14 days after nephrectomy and this period was selected for analysis in the radiation experiments. The dry renal tissue represents about 25% of total kidney weight and, in general, this percentage was unchanged during the course of kidney enlargement.

In the 2-month-old CF no. 1 mouse total DNA content of the normal kidney was 1.12 mg with a standard error of 0.05, whereas that of the kidney that had undergone enlargement for 5 or 6 days was 1.26 ± 0.06 mg (Table I). These differences are not significant. In both rat(5) and mouse(6) it has been reported that mitotic activity, if it occurs at all during the compensatory response, takes place within 48 to 72 hours after nephrectomy. Age appears to be an important factor since in the present experiments, DNA content was increased somewhat in 4- to 6-week-old mice but not in 2-, 4-, 6- or 10.5-month-old mice. A similar age-dependent relationship has been found in the rat(7).

TABLE I. Kidney DNA Content 5 to 6 Days After Unilateral Nephrectomy.

Age at operation	Group	No. kidneys	DNA/kidney (mg)
4-6 wk	Normal	13	.97
	Nephrectomized	13	1.50
2 mo	Normal	7	1.12
	Nephrectomized	7	1.23
2 " *	Normal	9	1.12 ± .05†
	Nephrectomized	9	1.26 ± .06
4 "	Normal	6	1.05
	Nephrectomized	6	1.08
6 "	Normal	3	1.16
	Nephrectomized	3	1.00
10.5 mo	Normal	11	1.03
	Nephrectomized	11	1.11

* Individual determinations; others are pooled values.

† Standard error of mean.

When the remaining kidney of a 2-month-old mouse is irradiated immediately after uninephrectomy, the hypertrophic response as revealed by the increase in either wet or dry weight at 14 days is not altered significantly until X-ray doses in excess of 20,000 r are employed (Table II). The weight of kidney exposed to 30 kr is significantly smaller than that of a nonirradiated compensating kidney although it is still greater than normal. The diminished response in this instance may be due, in part, to necrosis and scarring; however, even with 30 kr sufficient renal tissue persists to allow the animals to survive for several weeks. With 50 kr there is apparently considerable renal destruction and only 1 of 7 animals survived for 14 days. The increased

TABLE II. Effect of Local X-irradiation on Renal Enlargement After Unilateral Nephrectomy.

Group	Irradiation immediately after uninephrectomy			Irradiation 5 days after uninephrectomy		
	No. mice	Kidney wt (mg)*	P value	No. mice	Kidney wt (mg)*	P value
Compensating kidney						
0 r	18	188.2 ± 4.6	—	8	192.4 ± 6.1	—
10 kr	10	182.4 ± 4.0	>.05	9	169.7 ± 5.6	>.05
20 "	18	163.6 ± 9.3	<.05>.02	8	161.0 ± 10.4	.02
30 "	6	149.6 ± 8.9	<.001	6	136.9 ± 8.0	>.001
†Normal kidney	10	132.4 ± 4.0	—	—	—	—

* Mean ± S.E. 14 days after irradiation.

† Nonirradiated.

renal damage is probably reflected in the weight loss shown by the animals exposed to the higher dose levels. While the hypertrophic response of the kidney following uninephrectomy may not be affected by rather large amounts of radiation, there is evidence of latent injury in that mean survival time of such locally irradiated mice is reduced. This effect, however, is seen also in control mice in which both kidneys have undergone local irradiation: with 40 kr both groups showed a mean survival time of about 10 days; with 30 kr about a month; with 20 kr almost 3 months; with 10 kr about 3½ months.

The possibility exists that a kidney irradiated immediately after removal of the contralateral organ might be more or less sensitive than one undergoing hypertrophy at the time of exposure. To test this, mice were uninephrectomized, subjected to local kidney irradiation 5 days later, and sacrificed at 14 days. The picture is similar to that seen when irradiation occurs immediately after nephrectomy; the hypertrophic response shows a significant decrease at about the 20 kr level (Table II). It has been reported(6) that sublethal whole body X-irradiation 3 hours after unilateral nephrectomy suppresses mitosis and inhibits kidney enlargement in LAF_1 mice. However, if irradiation is delayed 48 hours, until mitosis has occurred, enlargement is essentially similar to that seen in nonirradiated controls. There are obvious differences of local vs whole body radiation effects as well as possible strain differences, but the discrepancy between these results and ours may also be attributable to the age-dependency noted previously.

Summary. In adult female CF no. 1 mice, kidney enlargement after unilateral nephrectomy is a consequence mainly of hypertrophy. New cell formation as evidenced by an increase in total DNA content of the kidney may be a contributing factor in young animals but this becomes negligible with increasing age. The compensatory response occurring after uninephrectomy in the adult mouse is quite radioresistant, whether irradiation occurs immediately or 5 days post-operatively. This contrasts with the well-known radiosensitivity of cell renewal systems.

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Metabolic Fate of Injected C^{14}-Phytosterols.*‡ (27076)

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It has been demonstrated(1-4) that plant sterols are absorbed by animals and man to a slight extent. The mechanism of phytosterol absorption has been investigated and shown to be similar to that of cholesterol(4). Several reports(1,2,5) have shown that following administration of H^3-β-sitosterol or C^{14}-ergosterol by feeding or intravenous injection labeled sterols could be detected in a number of tissues, the highest levels of activity being in the serum and liver. There is also evidence that fed H^3-β-sitosterol disappears more rapidly from the tissues and blood than cholesterol (1). Regarding the metabolic degradation products of the absorbed plant sterols, it has

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been shown that H^3-β-sitosterol is converted to H^3-cortisol in the guinea pig(5) and that C^{14}-ergosterol and H^3-β-sitosterol may be converted to bile acids(2,5). Thus far, it does not appear that fed or injected labeled plant sterols(1,2,5) are converted to cholesterol. In continuation of our studies(4) on plant sterols we have determined the comparative tissue distribution of injected C^{14}-phytosterols and cholesterol-4-C^{14}, their conversion to the esterified form in blood and tissues, and their conversion to bile acids.

Methods and Materials. Male rats weighing 225-250 g, of the Carworth strain, were divided into 4 groups; 2 groups were normal fasted rats which received intravenously (femoral vein) either 0.5 ml of an emulsion containing 0.34 mg cholesterol-4-C^{14} or C^{14}-phytosterols. The other 2 groups had bile fistulas and received similar emulsions. The source of the C^{14}-phytosterols was a crude lipid extract from the leaves of tobacco plants grown in a $C^{14}O_2$ atmosphere. The sterols were isolated and identified as described earlier(4). The labeled sterol emulsions were prepared by dispersing 0.34 mg of the labeled sterol in 0.5 ml of a solution containing 3 mg soybean lecithin in 0.05 M phosphate buffer pH 7.3(2). The animals were maintained under light anesthesia during the intravenous injection period (about 2 minutes). Bile was collected during the ensuing 24-hour period in the animals with bile fistulae. Twenty-four hours after injection of the labeled sterols the animals were sacrificed, and blood, liver, small intestine and adrenals removed. The small intestine was washed with saline and these washings were pooled with the feces of each animal. Lipid extracts of the tissues were prepared according to procedures described earlier(6). The bile samples (10-15 ml) were extracted with 10 volumes of ethanol and the sterols and bile acids separated(7). Free and total sterols were determined by the method of Sperry and Webb(8). The C^{14}-activities of the free and esterified tissue sterol fractions were determined as previously described(6). To ascertain if the injected C^{14}-phytosterols were converted to C^{14}-cholesterol, the sterols isolated from the several tissues were subjected to repeated passage through the dibromide and crystallization of the dibromides from 5:1 ether-acetic acid. This procedure has been shown to eliminate H^3-β-sitosterol from a sterol mixture containing cholesterol(5); the plant sterols do not form insoluble dibromides.

Results. The recovery of injected cholesterol-4-C^{14} and C^{14}-phytosterols (after 24 hours) in several tissues is shown in Table I. Of the tissues examined, the liver had the largest percentage of injected C^{14}-sterols. The percentage of injected C^{14}-sterols recovered as free C^{14}-sterol in the serum, liver and adrenals was the same for both the cholesterol-4-C^{14} and C^{14}-phytosterol groups. In the small intestine significantly ($P<.01$) less free C^{14}-sterol was recovered in the group injected with C^{14}-phytosterols than in the cholesterol-

TABLE I. Recovery of Injected Cholesterol-4-C^{14} and C^{14}-Phytosterols in Different Tissues after 24 Hours.

Tissue*	% Injected C^{14}-Sterol Recovered as Digitonide							
	Free		Ester		Total		% Ester	
	C^{14}-chol. group	C^{14}-phyt. group	C^{14}-chol. group	C^{14}-phyt. group	C^{14}-chol. group	C^{14}-phyt. group	C^{14}-chol. group	C^{14}-phyt. group
Serum†	2.32 ± .36§	2.50 ± .20	4.52 ±.72	2.74 ±.10	6.84 ±1.04	5.24 ± .30	66.1 ±2.0	52.3 ±3.0
Liver	20.88 ±4.14	20.82 ±2.50	2.12 ±.52	.96 ±.50	23.00 ±4.66	22.00 ±3.00	9.2 ±1.3	4.4 ±2.0
Small Intestine	3.60 ± .54	2.40 ±1.04	.24 ±.10	.08 ±.02	3.84 ± .64	2.52 ±1.01	6.3 ±1.5	3.2 ±1.0
Adrenals	.20 ± .02	.24 ± .06	.60 ±.14	.66 ±.08	.80 ± .16	.90 ± .16	75.0 ±1.6	73.3 ±2.3
Feces and Intestinal Contents	1.22 ± .40	4.04 ± .74	.10 ±.02	1.16 ±.26	1.32 ± .42	5.20 ±1.00	7.6 ±2.0	22.3 ±4.6

* Values represent average of 5 rats per group; each rat was injected with an emulsion containing either C^{14}-phytosterols (.34 mg; 61,000 CPM) or cholesterol-4-C^{14} (.34 mg; 243,000 CPM).

† Serum sterol content was estimated from blood volume and serum sterol concentration.

§ Standard deviation.

4-C^{14} group. The feces and intestinal contents of the C^{14}-phytosterol group had an amount of C^{14}-free sterol that was approximately 3 times greater than the group given cholesterol-4-C^{14}.

Esterified C^{14}-sterol was recovered from the tissues of animals injected with both types of C^{14}-sterols. The adrenal (with both injected C^{14}-sterols) had the highest percentage of esterified C^{14}-sterol of the tissues examined (73.3 and 75.0%). The total serum C^{14}-sterol fraction of both types of animals also contained a high percentage of esterified C^{14}-sterol, but the C^{14}-phytosterol group had significantly less (52.3%) than the cholesterol-4-C^{14} group (66.1%). The liver and small intestine of both types of animals had smaller amounts of esterified C^{14}-sterols than serum; also, less of the injected C^{14}-phytosterols was present in the esterified form in the liver and small intestine than when cholesterol-4-C^{14} was injected. Feces and intestinal contents of the group injected with cholesterol-4-C^{14} contained only a trace of esterified C^{14}-sterol; on the other hand, feces and intestinal contents of the C^{14}-phytosterol group contained 1.16% of the injected C^{14}-sterol as esterified C^{14}-sterol.

Both cholesterol-4-C^{14} and C^{14}-phytosterols were converted to bile acids (Table II); conversion of cholesterol-4-C^{14} to bile acid was twice that for C^{14}-phytosterols (14% vs 7%). The specific activities of the bile and liver free sterol fractions of the group given cholesterol-4-C^{14} were the same. In the C^{14}-phytosterol group a much larger proportion of the injected C^{14}-phytosterols was recovered as C^{14}-sterol in the bile than in the cholesterol-4-C^{14} group. Approximately 44% of the total C^{14}-activity recovered in the bile of the C^{14}-phytosterol group was present as bile sterol and only 9.2% in the group injected with cholesterol-4-C^{14}. The specific activity of the bile sterol of the C^{14}-phytosterol group was considerably higher than the liver free sterol fraction.

The free and esterified tissue sterols isolated from rats injected with C^{14}-phytosterols were subjected to 5 passages through the dibromide and crystallization of the dibromides from 5:1 ether-acetic acid. The specific activity of the tissue sterols of the C^{14}-phytosterol group declined to only about 1% of the original value, whereas, in the cholesterol-4-C^{14} group there was practically no change in the specific activity of the tissue sterols. These results would appear to indicate that very little, if any, conversion of C^{14}-phytosterols to cholesterol occurs.

Discussion. In many respects, C^{14}-phytosterols appear to follow the same metabolic pathways as cholesterol. They are selectively taken up by the liver, esterified by the liver, intestine and other tissues, and are converted to bile acids. However, there were several notable differences with respect to metabolism of the injected C^{14}-phytosterols. The degree of esterification of the injected phytosterols appeared to be less than that for cholesterol. It would also appear that while the C^{14}-phytosterols are removed from the serum by the liver as with cholesterol, they do not readily mix with the liver cholesterol pool and are selectively excreted in the bile. This is borne out by the specific activities of the bile and liver sterols of animals injected with C^{14}-phytosterols and also on the much greater proportion of injected C^{14}-phytosterols recovered as bile sterol. The data on the feces and intestinal contents also indicate that phytosterols are more rapidly excreted than cholesterol; the bile would appear to be the principal route of their excretion. The findings

TABLE II. Distribution of C^{14}-Activity in Bile Following Injection of C^{14}-Phytosterols and Cholesterol-4-C^{14}.

	Bile Sterol		Liver Free Sterol	Bile Acid
Group*	CPM/mg	% dose recovered	CPM/mg	% dose recovered
Cholesterol-4-C^{14}	2355 ±505	1.42 ±0.30	3163 ±450	14.06 ±3.05
C^{14}-Phytosterols	2269 ±647	5.46 ±1.56	615 ±150	6.98 ±2.10

* Values represent average of 5 bile-fistula rats per group; each rat was injected with an emulsion containing either C^{14}-phytosterols (.34 mg; 61,000 CPM) or cholesterol-4-C^{14} (.34 mg; 243,000 CPM).

with respect to conversion of C^{14}-phytosterol to bile acids confirm and extend the observations by Werbin *et al.*(5) and Hanahan and Wakil(2) with H^3-β-sitosterol and C^{14}-ergosterol. Preliminary experiments were carried out in an attempt to identify the bile acids formed. They appeared to be different from those derived from cholesterol.

Summary. Data are presented on the comparative tissue distribution of C^{14}-free and esterified sterols following injection of cholesterol-4-C^{14} and C^{14}-phytosterols to normal and bile-fistula rats. At the end of 24 hours a large proportion of the injected C^{14}-phytosterols and cholesterol-4-C^{14} was recovered in the liver. All tissues examined (serum, liver, small intestine and adrenal) showed the presence of esterified C^{14}-phytosterols, but less was esterified than with cholesterol-4-C^{14}. A much larger proportion of the injected C^{14}-phytosterols was recovered in the feces and intestinal contents than in the cholesterol-4-C^{14} group. Conversion of the injected C^{14}-phytosterols to bile acid was observed; a larger proportion of the injected C^{14}-activity was recovered in the bile as C^{14}-sterol than with injected cholesterol-4-C^{14}. No conversion of C^{14}-phytosterols to cholesterol or related sterols could be demonstrated.

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Destruction of Chimpanzee Kidney Cells by Sera From Patients with Acute Infectious Hepatitis.*† (27077)

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Recent epidemiological evidence(1) has suggested that chimpanzees may act as transient carriers of human hepatitis virus. Since then, recurrent outbreaks of the disease among groups of chimpanzee handlers have further supported this hypothesis(2-4). The outbreaks were sufficiently well documented to lend support to a supposition that the virus may have been propagated in some unknown cellular system within the animals.

Accordingly, chimpanzee liver, omentum, spleen, bone marrow, testis, and kidney were removed from healthy adolescent animals, and standard tissue culture techniques were employed to obtain cellular outgrowths. The various cellular systems were exposed to acute sera from 3 to 12 cases of typical human infectious hepatitis, suspected of containing active virus. Some of the sera were from chimpanzee handlers at Holloman Air Force Base, New Mex.(1), whereas others were derived from outbreaks remote from Holloman and not associated with any known ape contacts.

Initial experiments indicated that some of the sera were capable of destroying outgrowths of chimpanzee macrophages from omental tis-

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† Since this paper was submitted for publication, it has not been possible to maintain the virus past the 4th chimpanzee kidney cell passage.

sue. The destruction of monolayers of chimpanzee kidney cells by 2 of 12 sera, however, was so striking and the latter cells were so much more satisfactorily grown, that attention was focused on these cells. The destructive agents have subsequently been followed in serial passage in these cultures and partially characterized.

Methods. Sera. Five sera were obtained from certain chimpanzee handlers at Holloman Air Force Base who had typical cases of infectious hepatitis, ranging from 1 to 6 days after onset of icterus. Seven additional sera from hepatitis patients, who had not handled apes, were similarly collected from various Air Force installations and from civilian hospitals in San Antonio, Texas. Control sera were derived from normal, healthy Air Force personnel. Sera had been stored at −20°C from 1 to 10 months.

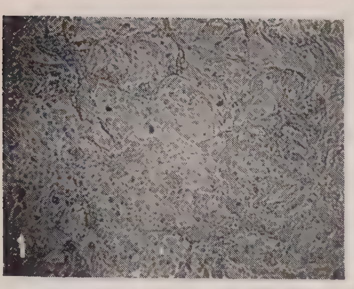
Kidney Cells. Kidneys were removed surgically from chimpanzees weighing 45 to 50 lbs and immediately subjected to overnight trypsinization according to procedures described by Youngner(5) and Bodian(6). After trypsinization and washing, cells were suspended in 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution(7) containing 5% calf serum (Microbiological Associates, Bethesda, Md.), 1% of a 10% $NaHCO_3$ solution, and penicillin and streptomycin (200 units/cc and 0.2 mg/cc, respectively), to a count of approximately 300,000 cells/cc. One cc volumes of the cell suspension were added to either screw-capped glass culture tubes or to Leighton tubes containing thin glass cover slips. Cultures were incubated, stationary, at 37°C, and medium was changed completely each 2 to 4 days, so that pH never fell below 7.0. When cellular growth was confluent, usually within 7 to 8 days, growth medium was removed, and the tubes were washed thoroughly with Medium 199(8) containing 0.1% $NaHCO_3$ and antibiotics as above (maintenance medium). One cc of fresh maintenance medium was then added to each tube. Following inoculation, tubes were maintained for 10 to 14 days at 37°C with the addition every 2 to 3 days of sufficient 10% $NaHCO_3$ to maintain pH above 7.0 (usually about 0.02 cc/tube).

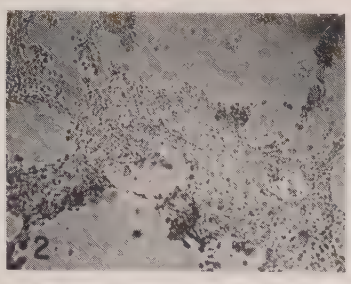
Inoculations. Each tube was initially inoculated with 0.2 cc of either acute hepatitis serum or "normal" serum. At least 4 tubes were inoculated with each serum in each of 2 initial experiments. In the case of sera producing destructive effects, serial passages were initiated by addition of 0.2 cc per tube of pooled fluid from frozen and thawed cultures which had shown extensive cellular destruction. Controls were derived from serial passage of fluids from cultures initially inoculated with "normal" sera.

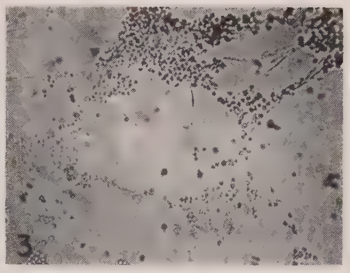
Results. Chimpanzee kidney cells inoculated with 2 of the 12 hepatitis sera showed evidence of cellular damage in initial cultures on the sixth day following inoculation (Fig. 1-3). One destructive serum was derived from Capt. JEC, Veterinary Services Branch, Aeromedical Field Laboratory, Holloman Air Force Base, a close contact of numerous immature chimpanzees. The second was obtained from PEL, a patient at the Stead Air Force Base Hospital, Nevada, who as far as could be ascertained, had no contact with chimpanzees.

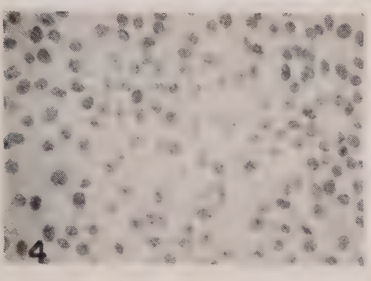
Cellular changes in fixed, stained preparations are illustrated in Fig. 4-6. By the seventh or eighth day after inoculation of the sera, destruction was complete. Original PEL serum, inactivated at 56°C for 30 minutes, similarly produced cellular destruction. Insufficient JEC serum was available for inactivation studies. Cells of the same age grown in serum from an individual with no known hepatitis showed no changes.

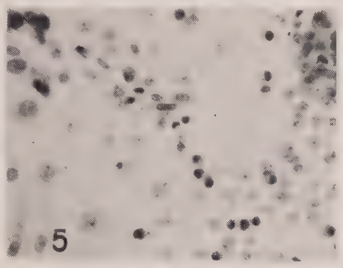
To characterize the destructive agents encountered in the 2 sera further, direct fluorescent antibody tests were performed on methanol-fixed cells 6 days after inoculation. Serum from Capt. JEC, 6 months convalescent from the acute episode of hepatitis, was labelled with fluorescein isothiocyanate according to methods outlined by Cherry et al.(9). Similarly, normal pooled serum from a group of 4 individuals at Holloman Air Force Base who had contracted no known hepatitis was labelled with fluorescein isothiocyanate for control purposes. Cells grown in JEC, PEL, and "normal" serum were fixed 6 days after inoculation, when destruction was still moderate. Each series was treated with labelled JEC serum and labelled normal serum pool. The fluorescent antibody test results are shown in Table I and Fig. 7-8.











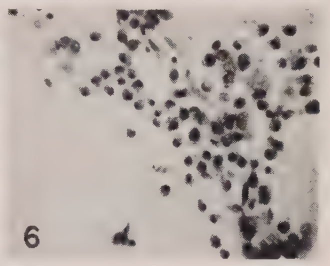


FIG. 1. Control chimpanzee kidney cells, grown in normal human serum for 6 days.

FIG. 2. Same as Fig. 1, except cells grown for 6 days in acute JEC serum.

FIG. 3. Same as Fig. 1, except cells grown in acute PEL serum.

FIG. 4. Chimpanzee kidney cells grown in normal human serum for 6 days, fixed and stained with hematoxylin and eosin.

FIG. 5. Same as Fig. 4, except cells grown in JEC serum.

FIG. 6. Same as Fig. 4, except cells grown in PEL serum.

TABLE I. Fluorescence of Chimpanzee Kidney Cells Grown in Acute Hepatitis Sera and Normal Serum Treated with Fluorescent Antibody.

	Fluorescein-labelled JEC Serum	Normal Serum Pool
Cells grown in JEC serum	+++	–
" " " PEL "	+++	–
" " " normal "	–	–

The destructive agents from JEC serum and from PEL serum, tentatively named SAM-1 and SAM-2, respectively, have at this time been carried through 3 serial tissue culture passages. Fluids removed from primary cultures 3 days after inoculation, when no destruction was evident, produced no change on serial passage. After the initial passage, cellular destruction occurred within 48-72 hours.

Agents SAM-1 and SAM-2, after first passage, survived heat treatment at 56°C for 30 minutes. Following first passage, both agents were titrated in chimpanzee kidney cells. Titers were in the range of only 1×10^{-2}. Nevertheless, the destructive effects of both agents, undiluted, were neutralized by JEC convalescent serum and not by "normal" serum.

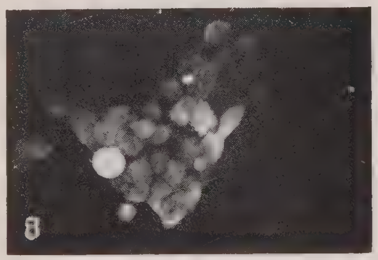


FIG. 7. JEC serum-infected cells (as in Fig. 2) treated with fluorescein-labelled hepatitis convalescent serum.

FIG. 8. PEL serum-infected cells (as in Fig. 3) treated with fluorescein-labelled hepatitis convalescent serum.

Discussion. The destructive effect of 2 acute hepatitis sera on chimpanzee kidney cells, capable of serial passage, suggests the action of a viral agent or agents. Fluorescent antibody and neutralization tests indicate that the agents are antigenetically reactive with convalescent serum from clinical infectious hepatitis. Heat inactivation studies further emphasize similarity to properties attributed to hepatitis virus. Studies to characterize the agents more clearly are presently in progress.

Summary. Destruction of chimpanzee kidney cells by 2 of 12 acute sera from patients with clinical infectious hepatitis was noted. The cellular effect has been seen in serial passage. The destructive agents, tentatively named SAM-1 and SAM-2, have been shown in fluorescent antibody and neutralization tests to be serologically cross-reactive with convalescent serum derived from clinical infectious hepatitis. The agents are resistant to heat treatment at 56°C for 30 minutes.

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Dose Dependent Effects of D-Sorbitol on Intestinal Absorption of Vit. B_{12}.* (27078)

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It has been shown that D-sorbitol enhances intestinal absorption of B_{12} under certain conditions in man(1) as well as in rats(2). Morgan and Yudkin(3) reported that growth of rats can be accelerated by supplement of sorbitol to a diet deficient in the B group vitamins. They postulated that sorbitol increased intestinal synthesis of these vitamins. Our study(4) also demonstrated that dietary sorbitol promoted growth of weanling rats placed on a B_6 deficient diet, with a concomitant increase in urinary output of B_6. No such effects were obtained with B_{12}, however. Inasmuch as increased synthesis of B_{12} in the intestine will not explain the enhancement of absorption of oral doses of B_{12}, a study was carried out in rats to elucidate the mechanism by which sorbitol improves B_{12} absorption. It was found that intestinal absorption of B_{12} could be either enhanced or reduced by coadministration of sorbitol, depending upon the dose. Such diverse, dose dependent effects of sorbitol will be reported herein.

Methods. Young adult rats of Wistar strain were used. Absorption of Co^{60} labeled B_{12} (Merck) was determined by one of the following methods. a) Absorption in intact rats: Animals were fasted overnight and then fed by a stomach tube a test dose of $Co^{60}B_{12}$, and absorption was estimated from the organ uptake of radioactivity. b) Absorption in ileostomized rats: In some animals, the ileum end was surgically severed and opened outside through the abdominal wall, the cecal end being closed. Thus, the cecum was excluded from the absorptive route, and absorption measured was that from the small intestine. The technique

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*The author wishes to thank Dr. B. F. Chow, Johns Hopkins School of Hygiene and Public Health, for his interest and advice in this work.

has been described elsewhere(5). c) Absorption by the "intestinal loop": A loop of the small intestine isolated *in situ*(5) was prepared to determine intrinsic factor activity of sorbitol and its effect on B_{12} absorption from the loop. $Co^{60}B_{12}$ was injected with or without rat stomach extract (intrinsic factor) into the loop which had previously been washed practically free of intrinsic factor, and radioactivity remaining in the loop after 24 hours was measured.

Four different dosages of $Co^{60}B_{12}$ were used; a single dose of 10 mμg(A), 50 mμg(B), and 2600 mμg(C), and 2 doses of 1000 mμg with a 24 hour interval(D). The radioactivity in each of these doses was approximately 0.01, 0.05, 0.15 and 0.1 mμcurie, respectively. The first dosage may be considered as being within physiological limits, and the latter 2 are supraphysiological. Crystalline D-sorbitol was dissolved in 1ml of distilled water mixed with 1ml of $Co^{60}B_{12}$, and administered. The animals were sacrificed 2 days later. Because of the diarrhea in sorbitol dosed animals, no fecal collection was attempted. Determination of radioactivity in organs with a gamma-scintillation counter has been reported(6).

Results. First, the effect of sorbitol on B_{12} absorption was studied in intact rats using doses A, B and D orally (Table I). It is apparent that coadministration of 600 mg of sorbitol markedly increased absorption of the 1000 mμg dose of B_{12}. The enhancement of absorption was less marked with decreasing levels of sorbitol, no further effect being observed below 100 mg (Studies 1 and 2). When the dose of B_{12} was lowered to 50 mμg, the effect of sorbitol to enhance absorption was less pronounced (Study 3). Upon further reduction of B_{12} dose to 10 mμg, this effect was no longer demonstrable. As a matter of fact, at this dose of B_{12}, the organ uptake of radioactivity in the animals given 600 mg of sorbitol showed a definite decrease. Thus, the effect of sorbitol was dependent on its dose as well as on the dose of B_{12}, and enhancement of absorption was marked only when a large amount of sorbitol was used in combination with a large dose of B_{12}.

Since the ceca of the rats receiving a large dose of sorbitol were invariably enlarged with liquid contents, it seemed of interest to investigate the role, if any, of the cecum in sorbitol-induced enhancement of absorption. In view of the finding that the effect of sorbitol was definite only when it was used in large quantities, 600 mg of sorbitol was used. Ten days after ileostomy, sorbitol and the test dose of $Co^{60}B_{12}$ were fed by mouth. The results (Table II) clearly showed that the effect of this compound to enhance absorption of a large dose of B_{12} was completely abolished. In fact, absorption was even reduced. Absorption of the physiological dose of B_{12} (10 mμg) was also decreased by sorbitol. Since the cecum did not take part in absorption in these animals, it

TABLE I. Effects of D-sorbitol on absorption of B_{12} and dose relationship.

Study	B_{12} Dosage	Group	No. of rats	Organ uptake of Co^{60} (as mμg B_{12}) Liver	Kidneys
1	1000 mμg x 2	Control	6	17 ± 1.6	19 ± 1.4
		Sorbitol (50mg)	4	18 ± 2.1	16 ± 1.8
		" (600 ")	4	31 ± 3.2	33 ± 6.5
2	idem	Control	4	24 ± 8.0	39 ± 6.7
		Sorbitol (100mg)	4	28 ± 5.4	46 ± 9.4
		" (400 ")	4	45 ± 9.0	95 ± 8.9
		" (600 ")	4	48 ± 7.1	116 ± 7.3
3	50 mμg x 1	Control	6	1.41 ± .12	.75 ± .11
		Sorbitol (600mg)	5	1.83 ± .16	1.00 ± .06
4	10 mμg x 1	Control	6	.51 ± .05	1.35 ± .16
		Sorbitol (50mg)	4	.48 ± .06	1.37 ± .20
		" (600 ")	6	.36 ± .04	.84 ± .10
5	idem	Control	4	.65 ± .06	.98 ± .09
		Sorbitol (600mg)	4	.42 ± .03	.65 ± .08

Figures are mean ± standard error.

TABLE II. Effect of D-sorbitol on B_{12} absorption in ileostomized rats.

Study	B_{12} dosage	Group	No. of rats	Organ uptake of Co^{60} (as mμg B_{12}) Liver	Kidneys
1	1000 mμg x 2	Control	4	76 ± 13.0	157 ± 33.4
		Sorbitol (600mg)	4	52 ± 7.5	51 ± 6.5
2	10 mμg x 1	Control	3	.82 ± .10	1.21 ± .15
		Sorbitol (600mg)	3	.50 ± .09	.69 ± .11

Rats were operated on 10 days prior to test.

seems that without this part of the intestine, sorbitol does not enhance absorption. Rather, large doses of sorbitol will reduce B_{12} absorption from the small intestine.

To corroborate these findings, the effect of sorbitol was studied further using the intestinal loop technique. When 350 mg of sorbitol was added to the test dose of B_{12}, absorption from the loop was reduced regardless of the dose of B_{12} (Table III). Addition of a stomach extract (intrinsic factor) increased absorption of 50 mμg of B_{12}. Sorbitol in a slightly hypotonic solution (70 mg/2 ml) had no similar effect. Neither did this dose of sorbitol reduce absorption. It is evident that sorbitol, under these conditions, is not capable of substituting for intrinsic factor, and that at its high concentrations, it interferes with absorption of B_{12} from the small intestine.

Discussion. Greenberg et al.(2) used a large dose of B_{12} when they first reported an increase of absorption by oral D-sorbitol in rats. In human studies, the intestinal absorption of 50 μg of B_{12} was repeatedly found to be increased by coadministration of sorbitol. This dose of B_{12}, too, is far from being physiological. Employing smaller doses of B_{12} in man, Herbert et al.(7) suggested inability of this compound to enhance physiological absorption of B_{12}. Chalmers and Shinton(8) failed to repeat the observations of earlier investigators using the same 50 μg of B_{12}, but the quantities of sorbitol fed were smaller. These differences in result seem to be explained on the basis that the effect of sorbitol varies with doses of both substances, as clearly demonstrated in this study. Our data showed that sorbitol exerts an influence on absorption only when it is used in large quantities; its effect is "enhancing" on supraphysiological doses of B_{12}, but it is "suppressing" on physiological amounts of B_{12}. It is established that absorption of supraphysiological doses of B_{12} does not require intrinsic factor. It is unlikely, therefore, that the effect of sorbitol is mediated by intrinsic factor. Sorbitol was found to possess no intrinsic factor activity as studied by the "loop" technique. The results obtained with ileostomized rats and intestinal loops indicated that sorbitol, in large quantities, inhibits B_{12} absorption from the small intestine which is the natural site of absorption. This was also reflected in the decrease of absorption of a physiological dose of B_{12} in intact animals as a result of sorbitol feeding.

That the ceca of sorbitol dosed rats were

TABLE III. Effect of D-sorbitol on B_{12} absorption from intestinal loop.

B_{12} dose (mμg)	Solutions applied to loop	Amt of sorbitol (mg)	No. of rats	Absorption (mμg)	Organ uptake of Co^{60} (mμg B_{12}) Liver	Kidneys
50	B_{12}	—	4	4.6 ± .9	1.04 ± .28	.89 ± .12
	" + Sorbitol	350	4	1.5 ± 1.2	.65 ± .09	.56 ± .05
50	"	—	4	4.5 ± .7	1.66 ± .12	.55 ± .04
	" + Sorbitol	70	4	3.9 ± .5	1.42 ± .31	.42 ± .09
	" + I.F.*	—	3	21.8 ± .8	5.17 ± .72	1.74 ± .30
	" + I.F.* + Sorbitol	70	3	18.8 ± .5	4.94 ± .64	1.80 ± .21
	" + I.F.* + Sorbitol	350	3	14.2 ± .5	3.26 ± .53	1.21 ± .18
2600	"	—	3	530 ± 65	126 ± 5	123 ± 11
	" + Sorbitol	350	3	320 ± 18	44 ± 5	60 ± 4

* Intrinsic factor in the form of rat stomach extract, containing B_{12} binding power of 80 mμg.

markedly enlarged and exclusion of this organ abolished the absorption enhancing effect of sorbitol seem to provide clues for elucidation of the mechanism. Hypertonic solutions of sorbitol act like a cathartic and increase the speed of descent of coadministered B_{12} in the digestive tract. Physiological absorption of B_{12} which is executed by the small intestine is reduced by accelerated passage(9). It is possible that an enlarged cecum with liquid contents alters the physiology of the intestine in such a way as to effect unphysiological absorption of B_{12} involving no intrinsic factor. Changes of intestinal flora and hence altered availability of B_{12} molecules to the mucosa may be an important factor.

The possibility still remains that a chemical conjugation of sorbitol to B_{12} as suggested by Heinrich(10) may be involved in an unknown fashion. As to the absorption efficiency of the mucosa for B_{12}, there is no evidence that sorbitol increases permeability of the mucosa. The data of the loop experiment suggested reduced permeability by a large dose of sorbitol. Whether the mechanisms working in the rat will directly apply in man awaits further investigation.

Summary. The effect of D-sorbitol on intestinal absorption of $Co^{60}B_{12}$ was studied in rats. The data demonstrated that sorbitol, when given by mouth in large quantities together with supraphysiological doses of B_{12}, enhanced absorption of B_{12} in intact animals. However, absorption of small, physiological doses of B_{12} was reduced by the same dose of sorbitol. The dependence of the sorbitol effect on the B_{12} dose may be explained on the basis of the 2 different mechanisms for B_{12} absorption. No intrinsic factor activity was demonstrated in sorbitol as studied with isolated intestinal loops. When the cecum was excluded from the absorptive route by ileostomy, the conditions that increased absorption in intact animals resulted in reduction of absorption. Possible involvement of the cecum and the altered physiological state of the intestinal contents in enhancement of B_{12} absorption by sorbitol have been discussed.

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Depletion and Synthesis of Fatty Acids in Chickens Fed a Diet Low in Unsaturated Fatty Acids. (27079)

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Linoleic (18:2)* and arachidonic (20:4) acids are considered to be essential fatty acids (EFA). Use of this term infers that either of these compounds will meet the dietary requirement for a fatty acid(s) which cannot be synthesized by the body in sufficient quantities for normal metabolism and growth. Although arachidonate may be the only fatty acid necessary for the function of the cell(1) it is not a specific dietary essential since linoleate is readily converted to arachidonate(2, 3). In contrast linoleate either cannot be synthesized sufficiently to be detectable by the methods used(4,5,6) or is synthesized in only minute amounts(7,8,9,10). The long periods of time involved in detecting symptoms of an EFA deficiency in the dog (12-40 weeks), rat (8-12 weeks), mouse (4 weeks) and guinea pig (4 weeks) (11,12,13,14) are usually interpreted as an indication of a slow rate of catabolism or excretion of EFA. In contrast to these species it has been observed(1) that in chickens, growth differences between EFA depleted and repleted groups can be detected in less than 2 weeks. Is this early response caused by a rapid decrease in tissue level of EFA due to catabolism of EFA or by rapid dilution of the EFA originally present with other fatty acids synthesized by the chick? In the present studies it was found that there is a rapid decrease in the proportion of 18:2 and 20:4 in tissues of chickens fed fat-free diets and this was largely a consequence of dilution of these acids with monoenoic and saturated fatty acids which were readily synthesized in the EFA-deficient chicken.

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* First number denotes chain length; the second, number of double bonds.

Procedure. The studies were conducted with Nichols 108 pullets reared in electrically-heated cages, maintained in an air-conditioned room provided with continuous illumination. In the first experiment day-old chicks were fed a low-fat diet containing 20% vitamin-free casein and 8% gelatin(1) for 7 days. They were then divided into 2 groups, one continued on the basal diet, the other the same diet except that 2% safflower oil plus 3.8% cellulose replaced 5.8% glucose. At various time intervals 3 to 5 chicks from each group were sacrificed and the heart and liver removed, frozen and stored at −15°C until analyzed for fatty acids by the gas chromatographic methods previously described(16). The results are expressed as percent by weight of total fatty acids excluding any with a retention time higher than that of 20:4.

In the second experiment a purified diet(1) containing 21% vitamin-free casein as the protein source and 1% methyl myristate as the only fat source was fed *ad libitum* for 28 days. In the third experiment the methyl myristate was deleted. The diets contain less than 0.005% linoleate and no detectable amount of arachidonate. The newly hatched chicks or 28 day-old chicks were sacrificed by putting them in a choloroform atmosphere and the entire carcass and feathers were ground in a large meat grinder until a relatively homogeneous mixture was obtained. The ground carcasses were frozen immediately and stored at −15°C until analyzed. Four grams of tissue were saponified by addition of 30 ml 25% KOH and heating at 50°C for 24 hours under nitrogen. The resulting solution was extracted 3 times with 10 ml portions of ether, brought to acid pH with concentrated HCl and extracted 3 times with 10 ml portions of pentane. The pentane extract was dried with anhydrous sodium sulfate, evaporated to dryness and weighed. This weight was considered to be total fatty acids. The percentage composition of the fatty acids was determined by gas chromatography by methods described previously(16). The grams of each fatty acid were calculated by multiplying total fatty acid content by percent of the particular fatty acid determined by gas chromatography(16). Four or five chickens from each treatment were analyzed.

Results and Discussion. The results are given in Table I. The change with time in

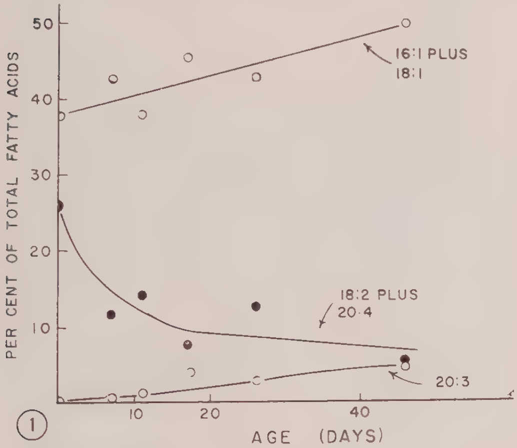
TABLE I. Change in Carcass Content of Fatty Acids with Age of Chickens Fed a Diet Free of Unsaturated Fatty Acids.

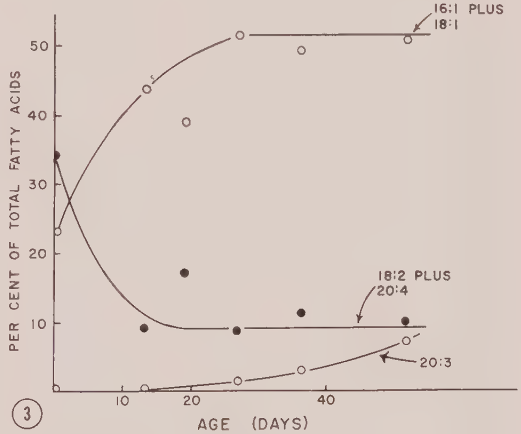
Fatty Acid*	Day Old %	Day Old mg	28-Day Old %	28-Day Old mg	Change mg
			Exp 1		
12:0	.0	0	.30	96	96
14:0	.6	18	13.60	3780	3762
14:1	.0	0	3.00	817	817
16:0	30.9	894	32.70	9030	8136
16:1	4.6	140	19.90	5470	5330
18:0	7.1	214	3.10	865	651
18:1	38.6	1165	26.80	7425	6270
18:2	16.4	517	.50	143	−364
20:4	1.4	39	.05	13	−26
TOTAL		2967		27639	24670
			Exp 2		
12:0	.1	2	.20	60	58
14:0	.4	9	1.90	571	562
14:1	.0	0	1.10	331	331
16:0	28.4	634	35.50	10675	10041
16:1	5.6	125	20.90	6284	6159
18:0	11.9	266	3.50	1052	786
18:1	37.9	847	35.50	10675	10614
18:2	14.6	326	.80	240	−86
20:4	1.0	22	.06	18	−4
TOTAL		2234		30070	27836

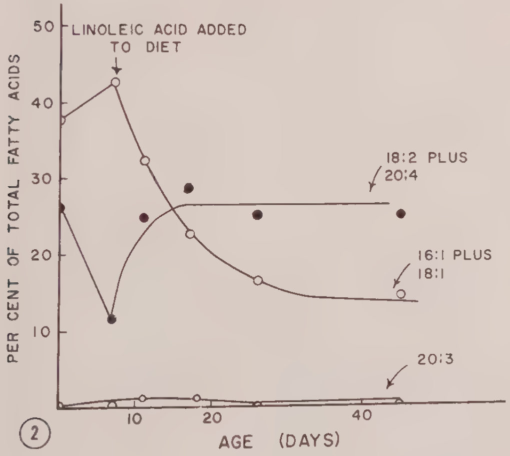
* First number denotes chain length; the second, number of double bonds. Analyzed by gas chromatography. Each number is average of at least 4 replicate analyses.

the proportions of fatty acids in livers of chicks fed EFA deficient diets is given in Fig. 1. Values for EFA (18:2 plus 20:4), the major monoenoic acids (16:1 and 18:1), and a fatty acid with a retention time compatible with a 20:3 structure are summarized. The decrease in the proportion of EFA is very rapid during the first 11 days and thereafter decreases quite slowly. As EFA decreased the monoenoic acids increased. This confirms the reciprocal relationship observed between 18:2 and the monoenoic acids(15). The 20:3 acid increased slowly but continually during the experimental treatment. When linoleic acid (as safflower oil) was added at 7 days EFA percent increased rapidly (Fig. 2). Within 4 days after addition of the linoleic acid (as safflower oil) to the diet the percentage of 18:2 and 20:4 was 16.0 ± 0.48% and 8.6 ± 0.82%, respectively, in the livers of chicks fed the basal diet. The differences between dietary treatments was statistically significant. The data again demonstrate the reciprocal relationship between EFA and the monoenoic acids. Similar results were obtained with heart tissue (Fig. 3 and 4).

During the 28 day growth period average fatty acid content of the chicken increased 24.7 g in Experiment 1 and 27.8 g in Exp. 2 (Table I). Most of this increase was the result of synthesis of saturated and monoenoic acids. Palmitate (16:0) was the predominant







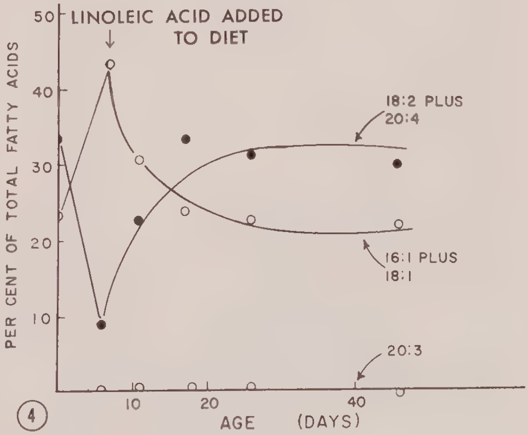


FIG. 1. Change in fatty acid composition of liver from chicks fed a low-fat diet.

FIG. 2. Effect of dietary linoleic acid on the fatty acid composition of liver.

FIG. 3. Change in fatty acid composition of heart from chicks fed a low-fat diet.

FIG. 4. Effect of dietary linoleic acid on the fatty acid composition of heart.

saturated fatty acid synthesized and palmitoleate (16:1) and oleate (18:1), the major monoenoic acids. This confirms the observation of Meade et al.(17), who found by use of a combination of silicic acid chromatography and alkaline isomerization procedures that the fats of rats fed EFA-deficient diets are rich in palmitoleate. The large amount (3780 mg) of myristate (14:0) recovered in the 28 day old chickens in Exp. 1 evidently was a result of deposition of dietary 14:0 since in the second experiment with no 14:0 in the diet only 571 mg was recovered in the 28-day old carcass.

In contrast to the saturated and monoenoic acids, the amount of 18:2 and 20:4 in the carcass of the 28 day-old chicken decreased to less than the initial amount present in the day old chickens. This suggests that there is some catabolism or excretion of 18:2 and 20:4. It is known that 18:2 is oxidized at a high rate by EFA-deficient mice(18), rats(19), and chickens(20). No data on 20:4 oxidation are available. Webb et al.(10) presented evidence for synthesis of 18:2 from acetate in fowl blood. However, based on the present studies such synthesis does not contribute significantly to the EFA economy of the young chicken.

As a result of the depletion of 18:2 and 20:4 concurrent with synthesis of saturated and monoenoic acids, the proportion of EFA in the fat of the body decreased drastically. In both experiments with 28-day old chicks the proportion of 18:2 or 20:4 was less than 1/16 of that of the newly-hatched chick. The rapid change in concentration of EFA may help explain why the chicken is sensitive to EFA deficiencies.

The chicken responds to supplements of EFA long before all 18:2 or 20:4 is depleted from the tissues(1). This suggests that merely a change in the proportion of 18:2 or 20:4 in some lipid structures may be sufficient to affect a critical function. Such functions may include regulation of enzymatic reactions(21, 22) or transport of critical metabolites in the blood. Addition of linoleic acid to a fat-free diet increases the deposition of short chain saturated fatty acid in adipose(1,23) and other tissues(24). This suggests another possible function for EFA, regulation of cell membrane permeability.

Summary. The proportion of linoleic and arachidonic acid in liver and heart tissue of chickens fed fat-free diets decreased to near minimal values within the first 2 weeks after hatching. Addition of linoleic acid to a fat-free diet at 7 days of age resulted in a significant increase of both linoleic and arachidonic acid in the liver of chicks fed the supplement within 4 days. Chickens fed a diet free of unsaturated fatty acids from 1 to 28 days of age synthesized over 24 g of fatty acids. This consisted mainly of palmitate, palmitoleate and oleate. During the same period the linoleate and arachidonate content of the entire body decreased, resulting in a reduction in the percent of these acids to less than 1/16 of that originally present in the newly-hatched chick.

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Encephalomyocarditis Virus Hemagglutination-Inhibition Test Using Antigens Prepared in HeLa Cell Cultures. (27080)

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Hemagglutinins can be demonstrated in suspensions prepared from the brain tissue of mice infected with the encephalomyocarditis (EMC) virus(1). Although cell cultures of many types support the growth of the virus (2), they have been utilized to only a limited extent for production of hemagglutinating (HA) antigen(3). During our investigations on occurrence of EMC hemagglutination-inhibition (HI) antibody in human sera, a need arose for large quantities of antigen. When reproducible results were not obtained with mouse brain suspensions, cell cultures were tried as a source of hemagglutinin. This report describes a method for preparation of EMC HA antigen in HeLa cells. Conditions necessary for optimal hemagglutination have been investigated and the findings applied to development of an HI test.

Materials and Methods. Virus. The EMC strain was recovered in this laboratory from the lung tissue of a naturally infected pig(4). It was isolated in HeLa cells and identified by neutralization tests employing a hyperimmune antiserum prepared against the American Type Culture prototype strain of EMC virus.‡ At the time of these studies the virus had received 5 consecutive HeLa passages.

HeLa Cells§ were grown as monolayers in 36 oz bottles in Eagle's medium containing 10% horse and 10% calf serum. Before virus inoculation cell sheets were washed twice with Hanks' solution; Eagle's medium containing 4% chicken serum was then added. Heated serum (56°C for 30 minutes) and antibiotics were routinely used in all media.

Diluents. The following solutions were prepared in distilled demineralized water: 0.15 M NaCl, 0.15 M KCl, 0.05 M H_3BO_3 in 0.12 M NaCl, 0.2 M Na_2HPO_4 − 0.2 M NaH_2PO_4 in 0.15 M NaCl (PO_4 buffer − NaCl) or 0.15 M KCl (PO_4 buffer − KCl) over the range pH 7.0 to 8.0, and 0.05 M H_3BO_3 in 0.12 M NaCl (BO_3 buffer − NaCl) or 0.12 M KCl (BO_3 buffer − KCl), adjusted with 1.0 M NaOH to pH 8.0 and 8.5. Occasionally, these solutions contained non-specific hemagglutinins which were readily removed by adsorption with small amounts of kaolin.

Red Blood Cells. (RBC) were collected from sheep, guinea pigs and humans in an acid-citrate-dextrose solution(5), and washed 3 times with isotonic saline. They were stored as a pack at 4°C for not longer than one week and resuspended in diluent as needed.

HA Tests. Four-tenths ml of antigen was serially diluted in 2-fold steps in 12 × 100 mm tubes. Four-tenths ml of a 0.4% suspension of washed RBC was then added and the mixture thoroughly agitated. Several different diluents were tried (Table I). Sedimentation was allowed to occur at 4°C or at 25°C and titers were read after 2 hours.

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‡ Kindly supplied by Dr. W. L. Pond, Nat. Inst. Allergy and Infect. Dis., Bethesda, Md.

§ Original seed obtained from Microbiological Associates, Inc., Bethesda, Md.

HI Tests. Serum was adsorbed with a 25% suspension of kaolin or extracted twice with acetone(5). To remove non-specific agglutinins serum was also adsorbed with packed RBC from the same source as those used in the test. Two-tenths ml of serum was diluted by 2-fold steps and 4-8 units of antigen in 0.2 ml was added.

After incubation of the antigen-serum mixture at room temperature for 1 hour 0.4 ml of a 0.4% suspension of RBC was introduced and the tubes thoroughly agitated. The antigen and RBC diluent are discussed below. Tests were read by the pattern technique following sedimentation at room temperature for about 90 minutes. Serum controls were included in all tests.

Virus Titrations. Serial ten-fold dilutions of virus were inoculated in 0.1 ml amounts into each of 2 HeLa tube cultures and endpoints were read after 5 days by cytopathic effect (CPE). Titers (TCD_{50}) were calculated by the method of Reed and Muench.

Results. Hemagglutinin Preparation. In attempts to demonstrate hemagglutinin in EMC-infected HeLa cell cultures, the medium overlying the cell sheets was HA tested at various times after the appearance of CPE. Hemagglutinin was usually not present in cultures exhibiting CPE, even though the fluid often contained moderate amounts of infectious virus ($10^{5.5} - 10^{6.5}$ TCD_{50}/ml). However, if the cultures were first subjected to alternate cycles of freezing and thawing, hemagglutinin at titers of 1:64 to 1:128 was readily demonstrated in the culture fluids. Because this suggested that physical destruction of infected cells was associated with the release of the hemagglutinating antigen, the following procedure was developed for routine preparation of antigen pools.

Monolayer bottle cultures were inoculated with 10^3 to 10^4 TCD_{50} of virus, a dosage known to initiate CPE in about 48 hours. When CPE was apparent cell sheets were washed with Hanks' solution and 50 to 60 ml of Eagle's medium without serum were added. After incubation for 6 hours at 36-37°C the cells and overlying medium were subjected to 2 quick cycles of freezing and thawing. This was accomplished by alternately immersing the bottle in baths of acetone-dry ice and 37°C water. The cell remnants and culture fluid were homogenized in a TenBroeck grinder and the preparation clarified by centrifugation. Supernatant fluids were stored at −20°C.

Diluents. Different solutions were tried with the purpose of determining whether the chemical composition or pH of the diluent influenced the hemagglutination reaction. Hemagglutination occurred at antigen dilutions greater than 1:4 only when diluents containing potassium (K) or boron (B) ions were used (Table I). Optimal results were obtained in the presence of both ions. The reaction appeared not to be pH dependent as it occurred in K or B solutions over a wide pH range. Interestingly, hemagglutination was not demonstrated in phosphate buffered solutions even when K or B ions were added. As a result of these findings, the BO_3 buffer-KCl solution adjusted to pH 8.0 was used routinely as the diluent in subsequent studies.

TABLE I. Effect of Chemical Composition of Antigen and RBC Diluent on Hemagglutination by EMC HeLa Antigen.

Antigen Diluent*	RBC Diluent*	Final pH	HA Titer‡
NaCl	NaCl	ND†	4
idem	PO_4 buffer—NaCl	7.0	<4
"	idem	7.5	<4
"	"	8.0	<4
"	PO_4 buffer—KCl	7.0	<4
"	idem	7.5	<4
"	"	8.0	<4
"	BO_3 buffer—NaCl	8.0	4
"	idem	8.5	<4
H_3BO_3—NaCl	NaCl	ND	8
BO_3 buffer—NaCl	PO_4 buffer—KCl	8.0	<4
idem	NaCl	8.0	8
"	BO_3 buffer—NaCl	8.0	16
"	KCl	8.0	64
KCl	idem	ND	16
idem	BO_3 buffer—NaCl	8.0	64

* Diluent composition and concentration defined in text.

† Not done.

‡ Expressed as reciprocal of antigen dilution.

RBC. It has been reported(1,6) that optimal results with EMC mouse brain antigens were obtained by using sheep RBC at low concentrations (0.25%) and overnight sedimentation of cells at 4°C. Since in our locality sheep RBC were not readily available, guinea pig and human Type "O" erythrocytes were tried. Titers with these cells did not substantially differ from those obtained by us with sheep RBC. HA tests employing guinea pig or human RBC gave similar results at sedimentation temperatures of 4° and 25°C. While guinea pig RBC were used in most experiments, human "O" cells were found to be useful in HI tests of human serum because adsorption for nonspecific agglutinins was not necessary. The HI testing of large numbers of sera was facilitated by using RBC concentration of 0.4% and carrying out tests at room temperature, since it was possible to read endpoints after cell sedimentation for 90 to 120 minutes.

Hemagglutinin Stability. The antigen was stable at pH 8.0 in the BO$_3$ buffer-KCl diluent: no significant change in titer was noted after several months' storage at -20°C, up to 8 weeks in the refrigerator at 4°C and after standing overnight at room temperature.

Serum Inhibitors. In HI tests with human and swine sera known to be free of EMC-neutralizing antibodies, naturally occurring inhibitors were frequently found. Removal of these inhibitors was readily accomplished by treatment with kaolin but not by acetone extraction (Table II). HI titers were easily read after kaolin treatment of serum since hemagglutination patterns were smooth and slippage of cells rarely occurred.

TABLE II. Removal of Non-Specific EMC Hemagglutination Inhibitors by Kaolin but not Acetone.

Serum		Treatment	
	None	Kaolin	Acetone
Human	16*	<8	32
	64	<8	16
	8	<8	Sl†
Swine	32	<8	16
	8	<8	8
	16	<8	16
	16	<8	16

* Reciprocal of serum dilution completely inhibiting hemagglutination by 4 units of antigen.

† Slippage.

HI Tests. We have employed the methods described to survey human sera for EMC antibody and to follow the serological response of swine experimentally infected with the virus. Of 33 human sera shown to possess EMC neutralizing antibody, 19 were found to have HI titers of 1:4 or greater. Titers of 1:64 or higher were demonstrated in several of these sera. Only one of 35 sera negative by neutralization test had HI antibody. HI antibody was found in the serum of swine as early as 8 days following the oral administration of EMC virus. In these animals an increase in HI titer was associated with the appearance of serum neutralizing antibody.

Discussion. Jungeblut and Kodza(7) were unable to obtain HA titers greater than 1:4 with the Col SK strain of EMC virus adapted to HeLa cells. These workers suggested that the relatively low concentration of infectious virus in the medium overlying the cell sheet ($10^{5.5}$ in tissue culture fluids as compared to 10^8-10^9 in the mouse brain) accounted for the low HA titer. Our prelimintary tests using culture fluids as antigens gave similar results. Antigen pools with high HA titers were regularly obtained only when the cells exhibiting CPE were alternately frozen and thawed.

Suspensions of EMC infected mouse brain have been used to determine some of the physical and chemical conditions necessary for hemagglutination(1). This reaction was found to occur optimally in the presence of K but not Na or the divalent cations, Mg and Ca. Using tissue culture antigen, we confirmed the K ion requirement. It is of interest that hemagglutination was also demonstrated in presence of the trivalent B ion. The diluent adopted for routine use in the HI test gave consistently satisfactory results and made possible standardization of pH without phosphate buffer. Gard(6) has reported that PO$_4$, as well as several other polyvalent anions, inhibits EMC hemagglutination. Our tissue culture antigen did not hemagglutinate in phosphate buffered solutions even when K or B were added.

The EMC HI test described here offers certain definite advantages over previously reported methods(1,6). Large amounts of a relatively "clean" antigen can be prepared in tissue cultures. The use of higher concentrations of RBC and room temperature for sedimentation makes the test quicker to perform and less cumbersome. Kaolin adsorption of serum adds to the clarity of results and effectively removes non-specific inhibitors. In our hands, the test has been no more time consuming or difficult than procedures now employed to detect myxovirus HI antibodies(8). Preliminary results suggest that the HI test can be utilized for purposes of diagnosis and population survey.

Summary. 1. A stable EMC hemagglutinating antigen was prepared by alternate freezing and thawing of virus-infected HeLa cell cultures. 2. Optimal hemagglutination titers were obtained at 4° and 25°C with human and guinea pig erythrocytes when diluents containing potassium and boron ions were used. 3. A hemagglutination-inhibition test employing kaolin treated serum and tissue culture antigen is described and its practical usefulness in detecting antibody in human and swine serum is demonstrated.

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Quantitative Aspects of Friend Leukemia Virus in Various Murine Hosts.*† (27081)

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The leukemia virus described by Friend (FV) is considered to have a narrow and specific adult murine host range(1,2,3), severely limiting the type of experiments which may be carried out. This virus has a relatively short latent period characterized by splenomegaly about one week after inoculation, with blood changes occurring later in the disease. Because of the obvious advantages of utilizing a virus producing early onset of overt disease, it seemed desirable to reinvestigate the host range with particular attention to the reliability of splenomegaly as an early indicator of infection and susceptibility. It was thought possible that inapparent infection might occur in some strains of mice without the usual early pathognomonic signs.

Materials and Methods. C57BL/Crgl, C3H/Crgl, C3H/Crgl/2, DBA/2NCrgl, A/Crgl mice were obtained from Cancer Research Genetics Laboratory, Univ. of California, Berkeley. BALB/c mice were either from our own colony or Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and are so indicated. The random bred Swiss mice were from the colony of the Hooper Foundation. All animals were 6-8 weeks old at time of inoculation.

The Friend virus used in these studies was received from Dr. C. F. T. Mattern of the Virus Laboratory, Univ. of California, Berkeley. He had received it from Dr. Charlotte Friend after an unstated number of passes in Swiss mice. The stock virus used was from a large pool of spleens prepared from the 11th pass in Swiss mice in this laboratory. Spleens were homogenized in a sucrose-phosphate buffer solution as a 20% suspension. This was centrifuged at 2,000 rpm for 10 minutes and the supernate distributed in sealed ampules

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and stored at − 70°C. Before use the suspension was recentrifuged at 2,000 rpm for ten minutes.

To determine susceptibility, mice of each strain were injected intraperitoneally with 0.5 ml of the 20% suspension of virus. At 14, 21 and 28 days after inoculation 7 to 10 mice from each group were sacrificed, the spleens removed, weighed, and a piece of each taken for histologic examination. Those showing foci of proliferating reticulum cells were deemed infected. The remainder from each group was pooled and treated the same as the stock virus. All spleen suspensions were diluted decimally from 10^{-1} to 10^{-6} and each dilution inoculated into groups of 10 Swiss mice except as otherwise indicated. Deaths occurring prior to the 8th day after inoculation were considered to be non-specific. All animals dying after this time and those sacrificed at termination of the experiment on the 35th day were autopsied. Those with spleens weighing greater than 0.5 g were considered to be infected with FV and were not examined further on the assumption that histologic evidence of disease was also present. An ID_{50} was then calculated on the basis of overt disease. Those with spleens weighing less than 0.5 g were examined histologically and the ID_{50} was recalculated from the combined total of those showing overt disease or microscopic evidence of infection.

Spleen weights were also obtained from uninoculated mice of each strain.

During the latter stages of this work Dr. Friend kindly supplied us with 2 ampules of lyophilized virus passed in her laboratory. Our own BALB/c and Swiss mice were tested for susceptibility to this virus.

Results and Discussion. It is apparent from Table I that the strains of mice fell into 3 categories of susceptibility to FV. The A and C57BL showed neither splenomegaly nor histologic evidence of disease. Both groups of C3H represented an intermediate stage in that approximately 58% showed some evidence of infection. The DBA/2, BALB/c and Swiss mice appeared to be equally and maximally susceptible, 96 to 100% showing evidence of FV disease.

We have examined several thousand mice and have only rarely encountered a normal animal with a spleen weight of greater than 0.5 g, and in this respect splenomegaly was a reliable indicator of infection. However, it is obvious from Table I that a number of animals may be infected with FV without splenomegaly. This was particularly true among animals of intermediate susceptibility and in the early stages of disease.

These results appeared to be at variance with those reported by Friend concerning the limited mouse strain susceptibility. However, the inoculum given our mice was 5 times greater than she administered. Since this could have accounted for infection in the C3H and BALB/c mice, quantitative studies were carried out. From Table II it may be seen that the animals still fell into 3 well defined groups.

TABLE I. Relative Susceptibility of Various Strains of Mice to Infection with Friend Virus as Determined by Splenomegaly and Microscopic Examination.

Strain	Normal Spleen wt (avg)	Results at indicated time after intraperitoneal inoculation.								
		14 days			21 days			28 days		
		Spleen wt (avg)	No. Infected/Total		Spleen wt (avg)	No. Infected/Total		Spleen wt (avg)	No. Infected/Total	
			Spleen wt*	Histo†		Spleen wt*	Histo†		Spleen wt*	Histo†
A/Crgl	.11	.24	0/10	0/10	.26	0/10	0/10	.28	0/10	0/10
C57BL/Crgl	.08	.21	0/9	0/9	.23	0/10	0/10	.29	0/10	0/10
C3H/Crgl	.12	.42	2/7	4/7	.71	4/7	4/7	.53	1/7	4/7
C3H/Crgl/2	.12	.34	1/10	4/10	.49	4/9	5/9	.49	4/10	8/10
DBA/2NCrgl	.10	.43	2/8	8/8	1.11	9/9	9/9	.95	10/10	10/10
BALB/cJax	.17	.55	4/8	7/8	1.99	9/9	9/9	2.80	9/9	9/9
BALB/c	.21	.64	5/9	8/9	1.66	9/9	9/9	2.07	10/10	9/10
Swiss	.22	1.19	8/10	10/10	2.02	9/10	9/10	1.08	9/10	10/10

* Spleens weighing >.5 g were considered infected.

† Spleens examined histologically and those showing foci of proliferating reticulum cells were considered infected.

TABLE II. Multiplication of Friend Virus in Various Strains of Mice as Determined in Swiss Mice.

Strain	Titer of virus from spleens harvested at indicated time after inoculation†					
	14 days		21 days		28 days	
	Gross*	Combined‡	Gross*	Combined‡	Gross*	Combined‡
A/Crgl	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
C57BL/Crgl	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
C3H/Crgl	<1.0	<1.0	2.8	3.1	2.5	2.8
C3H/Crgl/2	1.4	1.4	2.7	2.8	2.3	2.9
DBA/2NCrgl	3.0	3.8	3.7	4.4	3.7	4.4
BALB/cJax	3.5	4.4	3.9	4.3	3.9	4.5
BALB/c	3.5	4.6	4.4	4.8	4.2	4.6
Swiss	3.0	3.6	4.0	4.6	2.5	3.1

† $-\text{Log}_{10}$ of ID_{50} per .5 ml.

* Spleens weighing $>.5$ g were considered infected.

‡ Combined titer calculated from total of spleens weighing $>.5$ g or showing histologic evidence of disease.

The virus apparently failed to multiply in the A and C57BL strains. Although the titer was less than 10^{-1} an occasional Swiss mouse inoculated with spleens from these animals showed evidence of disease. The virus multiplied slowly and at a low level in the C3H mice, reaching a peak by 21 days and maintaining this level through the 28th day after infection. The DBA/2 and BALB/c mice appeared to be maximally and slightly more susceptible than the Swiss mice. The virus titer reached its peak in the first 2 strains between 2 and 3 weeks and was maintained through the 4th week, while in the Swiss mice there appeared to be a decline in virus titer between the 3rd and 4th week. It is obvious, however, that at 21 days no significant differences could be observed in the susceptibility of these 3 strains of mice. The differences between the titer of virus as determined by gross examination and that determined after histological examination of the spleens weighing less than 0.5 g were not of statistical significance. Nonetheless, in the DBA/2, BALB/c and Swiss mice the titer was consistently higher after microscopic examination than when splenomegaly alone was depended upon as an index of infection. Animals which were positive only on histologic examination were found in all dilutions, indicating that this phenomenon was not entirely dependent upon dose of virus or incubation period. Since these titrations were carried out in Swiss mice and there exists an inherent resistance in 10 to 20% of these mice to overt disease(1,2), these results were not unexpected. The inbred C3H mice also exhibited a high degree of resistance to overt disease. Of the 33 C3H mice of both strains which were examined 3 and 4 weeks post inoculation, 30% had overt disease while 64% had histologic evidence of infection. In one group of these mice sacrificed 63 days after inoculation only 4 out of 10 had splenomegaly while 9 showed microscopic evidence of disease.

Since it was desirable to assay virus in mice of more uniform susceptibility, BALB/c mice were investigated further. Two separate lots of virus prepared from BALB/c spleens after 1 and 2 passes in these animals revealed gross titers of $10^{-3.6}$ and $10^{-4.8}$ respectively, as assayed in BALB/c mice. Among the animals without overt disease in these titrations only 2 mice were found to be positive microscopically, both of them in the 10^{-4} dilution of the first titration. Futhermore all 60 mice inoculated with dilutions 10^{-1} through 10^{-3} developed clinical infections which is in contrast with the irregular results attained in Swiss mice. Additional passage of BALB/c adapted virus in BALB/c mice failed to reveal resistant mice when these were inoculated with 0.2 ml of 10% mouse spleen suspension. At the 13th passage level all of 69 BALB/c mice showed overt signs of disease when sacrificed 20 days after inoculation of FV.

Since Friend had reported BALB and C3H mice to be resistant to infection with this virus (1,3), it seemed possible that our strain was either a variant or another virus. One of the ampules of virus received from Friend was diluted to 1 ml with water to make a 10% suspension and was inoculated intraperitoneally in 0.2 ml amounts into 5 Swiss mice. The mice were sacrificed 20 days after inoculation and only one had splenomegaly. The spleens of these mice were made into 10% suspension and inoculated in 0.2 ml amounts into 30 Swiss and 10 BALB/c mice. They were sacrificed 17 days later and 90% of the Swiss and 70% of the BALB showed splenomegaly. Microscopic examination of the enlarged spleens from both Swiss and BALB/c mice revealed changes identical to those observed by us in Swiss, BALB/c, DBA/2 and C3H mice inoculated with virus previously passed in our laboratory.

Virus could not be isolated from the second ampule after 2 blind passes in BALB/c and Swiss mice.

Summary. The murine host range of Friend leukemia virus has been investigated quantitatively. Virus failed to multiply in C57BL and A strains. About 70% of C3H mice were susceptible, but overt disease developed in only 39%, the remainder being detectable by histologic examination only. Virus multiplied more slowly and to a lesser degree in C3H mice than in Swiss, DBA/2 and BALB/c, which were highly susceptible. Splenomegaly was more marked and more regular in BALB/c than in any of the other strains investigated; 100% of these mice being susceptible after initial adaptation.

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A Shortened Dental Caries Assay with the Cotton Rat.* (27082)

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One of the handicaps of dental caries research has been the lack of adequate means of evaluation of the process. Use of expermental animals in which actual initiation and progression of carious lesions are studied has been most helpful. Shaw *et al.*(1) devised an assay using the cotton rat. This animal has been used almost exclusively in this laboratory and has provided much data on dental caries in relation to the diet. However, for rapid progress required in the isolation studies, it was tedious and time-consuming. Shaw(2) has emphasized the importance of heredity in dental caries susceptibility. Constance *et al.*(3) found that withholding the feeding of a cariogenic diet until the teeth of the cotton rats were more mature, decreased their susceptibility to the caries process. Taketa *et al.*(4) demonstrated that the influence of the maternal diet was far less important than that of the diet fed the young cotton rat. With these observations at hand, it seemed that the calcification process was the fundamental point involved, that the early transfer of suckling young from mother to a cariogenic diet would enable us to reduce the time factor involved in the cotton rat assay method, and provide information less subject to individual variation caused by hereditary or other factors.

Experimental. Cotton rats, 16-19 days old were housed individually in wire mesh cages and given feed and distilled water *ad libitum.* The length of the experimental period was either 10, 30 days or 12 weeks. For the 10 and 30 day assay, animals were weighed only at the end of the experimental period and for the 12 week assay, they were weighed weekly. The animals were sacrificed and their teeth evaluated for caries according to the method of Shaw *et al.* For the purpose comparing the assay period, the 802 cariogenic diet and the 802 + 25% oat hulls non-cariogenic were used.

Results. A 10-day assay period was insufficient to develop and demonstrate carious le-

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sions. At the end of 30 days, the carious lesions were sufficiently evident to allow evaluation. It is evident from the data presented in Table I that there was a marked difference in incidence score between the teeth of the rats fed the cariogenic diet 802 and those fed 802 with added oat hulls. The caries incidence for the 30 day period was equal to that of the rats assayed at 12 weeks. The extent, or enlargement of the lesions was reduced with the use of the 30 day assay. It was observed that the initial age of transfer to the experimental diet was the most critical factor involved (Table II). It was necessary to confine the initial age of the young rats to 16 and 17 days of age in order to obtain consistent and reliable results with the 30 day assay. One difficulty was encountered in that a 16 day old rat may not acclimate himself to the expermental regimen and refuse to eat. If such an animal persists in feed refusal, it should be replaced. Growth rate during an experiment is most important. Animals that fail to grow should be eliminated from the assay.

TABLE I. Comparison of 12 week and 30 day assays of a cariogenic and a noncariogenic diet.*

Assay	Diet	No. of animals	Caries score Incidence	Caries score Extent
12 wk	802	37	22	46+
	802 plus 25% oat hulls	36	10	13+
30 day	802	39	20	25+
	802 plus 25% oat hulls	23	6	7+

* 802 was considered the cariogenic diet and 802 plus 25% oat hulls the noncariogenic diet.

TABLE II. Effect of initial age of cotton rats upon caries production at the end of 30 days.

Initial age days	Caries score for 802 diet Incidence	Extent
16-17	20	25+
18-19	13	17+

Summary. A 30 day cotton rat assay was developed which gave results comparable to those obtained with rats assayed at 12 weeks. Age and growth rates were found to be factors which greatly affect the assay. Age and maturation of the tooth account for much of the biological variation encountered in experimental dental caries production and assay.

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AUTHORS' INDEX

VOLUME 108

(The numerals indicate the page)

Abe, T. 234.
Ahrens, E. H., Jr. 286.
Aikawa, J. K. 684.
Albaum, H. 569.
Alexander, C. S. 706.
Alexander, H. E. 755.
Alger, E. A. 484.
Allen, E. G. 109.
Alt, H. L. 793.
Ambrus, J. L. 360.
Amiraian, K. 454.
Anderle, S. K. 183.
Andrews, M. 619.
Angeletti, P. 53.
Annable, C. R. 238.
Anthony, W. L. 555.
Antopol, W. 569, 587.
Aramaki, T. 242.
Arndt, W. F. 166.
Arnow, P. M. 170.
Arredondo, M. I. 216.
Astrop, T. 369.
Aulisio, C. G. 56.
Axelrod, B. J. 592.
Axelrod, A. E. 549.

Back, N. 360.
Bailey, W. L. 774.
Baker, B. L. 238.
Baker, G. D. 555.
Barker, J. N. 803.
Barnes, R. H. 468.
Barron, K. D. 71.
Bartlett, P. 611.
Bavetta, L. A. 38.
Beasley, R. M. 634.
Beher, M. E. 555.
Beher, W. T. 555.
Behrman, S. J. 645.
Benjamini, E. 700.
Berg, R. B. 616, 742.
Berger, A. 661.
Berger, H. 676.
Berman, L. 434.
Bernard, R. A. 784.
Bernecky, J. 347.
Bernsohn, J. 71.
Birnbaum, D. 321.
Bishop, C. 192.
Björklund, B. 385.
Björklund, V. 385.
Blair, P. B. 289.
Blankenhorn, D. H. 43.
Blaquier, P. 711.
Blinick, G. 569.
Boccabella, A. V. 484.
Bohr, D. F. 711.
Bolognani, L. 70, 111.
Bostick, W. L. 826.
Bowman, D. C. 432.
Boyce, W. H. 726.
Bradley, J. E. 425.
Bradley, S. G. 79.
Brahmankar, D. M. 337.
Breibart, S. 697.
Brooks, C. McC. 520.
Brostoff, D. 674.
Broughton, C. T. 198.
Brzezinski, A. 342.
Bumin, I. 82.
Burke, E. C. 276.
Burkhalter, A. 735.
Buynak, E. B. 508.
Byerrum, R. U. 162.

Campbell, T. N. 529.
Campillo-Sainz, C. 67.
Cangelosi, J. 121.
Castor, C. W. 574.
Celada, F. 129.
Chang, T.-W. 471.
Chanock, R. M. 56.
Chapman, C. L. 372.
Chapman, J. S. 566.
Chow, K. L. 92.
Chryssanthou, C. 587.
Clabaugh, W. A. 216.
Clapp, H. W. 238.
Clem, L. W. 762.
Coccheri, S. 369.
Cohen, S. 119.
Collison, H. A. 641.
Conway, E. 629.
Cook, J. E. 372.
Cooper, G. W. 546.
Cooper, J. A. D. 793.
Copeland, W. H. 117.
Correa, J. 336.
Craighead, J. E. 823.
Cramer, J. W. 105.
Cranston, E. M. 514.
Crastnopol, P. 82.
Cristoffanini, A. P. 689.
Crout, J. R. 482.
Cutting, W. 244.

Damm, H. C. 144.
Daniel, L. J. 119.
Daniels, J. B. 805.
Danon, A. 342.
Danowski, T. S. 559.
Davidson, E. A. 533.
Davis, J. H. 252.
Davis, R. B. 774.
Dawson, P. J. 826.
Dayton, S. 257.
Dear, W. 377.
DeEds, F. 623.
DeLuca, H. F. 105.
DeMaria, W. J. A. 122.
DeOme, K. B. 289.
Detwiler, T. C. 428.
Dewey, M. M. 238.
Dickinson, J. C. 760.
Didakow, N. C. 768.
Diller, E. R. 637.
Dinning, J. S. 366.
Doemling, D. B. 432.
Dormandy, T. L. 597.
Dorstewitz, E. L. 574.
Douglas, A. 82.
Dragstedt, L. R., II. 92.
Drucker, W. R. 99.
Dubes, G. R. 412.
Dutton, A. M. 786.
Dvorak, H. F. 564.

Eaton, M. D. 766.
Edgington, T. S. 149.
Egge, A. S. 198.
Eigelsbach, H. T. 732.
Enders, J. F. 406.
Endröczi, E. 154.
Engstrom, G. W. 105.
Epstein, W. V. 744.
Ercoli, A. 3.
Ershoff, B. H. 160.
Ertug, N. 82.
Essig, H. W. 194.
Evans, C. A. 776.
Everett, J. W. 604.

Faarvang, H. J. 591.
Falconi, G. 3.
Feingold, B. F. 700.
Feldstein, A. 308.
Fellows, J. 592.
Fielden, M. L. 726.
Fieldsteel, A. H. 826.
Finder, A. G. 248.
Fine, J. 261, 479, 709.
Finger, K. F. 680.
Fishman, V. 178.
Follis, R. H., Jr. 136.
Forbes, G. B. 697.
Forbes, M. 34.
Forney, R. B. 157.
Fox, M. R. S. 703.
Fox, R. R. 663.
Frank, E. 261.
Frank, E. D. 709.
Franklin, R. M. 651.
Fraps, R. M. 291.
Fried, M. 529.
Frothingham, T. E. 616.
Funderburk, W. H. 680.
Furchner, J. E. 797.
Furusawa, E. 244.

Gal, E. M. 160.
Gama, M. del. 67.
Gann, D. S. 99.
Garner, G. B. 512.
Gaston, E. O. 486.
Gayle, R. 53.
Gedalia, I. 342.
Geiringer, E. 658.
Gerber, P. 205.
Gershowitz, H. 645.

Geyer, R. P. 6.
Ginzburg, L. 569.
Goldberg, A. 535.
Goldberg, L. G. 340.
Goldenberg, H. 178.
Gomatos, P. J. 651.
Gonzales, F. 134.
Good, R. A. 79.
Grace, J. T., Jr. 360.
Gresser, I. 303, 799.
Griffith, G. D. 162.
Griffith, W. R. 732.
Grose, F. 619.
Grubbs, R. E. 205.
Gruenewald, R. 109.
Gubler, K. 674.
Guillemin, R. 377.
Guze, L. B. 496.

Haight, A. S. 209.
Halevy, S. 6.
Hall, T. 321.
Halpern, B. P. 784.
Hamdy, M. K. 185, 189.
Hamilton, C. L. 319.
Hammond, C. W. 183.
Hamparian, V. V. 444.
Harding, H. R. 96.
Hardwick, B. C. 402.
Harms, D. R. 684.
Harris, S. C. 432.
Hartley, J. W. 390.
Harvey, O. A. 637.
Hayes, R. O. 805.
Hazelwood, B. S. 10.
Hazelwood, R. L. 10.
Herion, J. C. 493.
Hess, A. 71.
Hilleman, M. R. 444.
Hilliard, J. 154.
Hillis, W. D. 813.
Himeno, K. 627.
Hinshaw, L. B. 24.
Hiramatsu, M. 315.
Hiramoto, R. 347.
Hoffman, A. J. 634.
Hofmann, K. 559.
Hogue, D. E. 77.
Hollander, W. P. 600.
Homburger, F. 152.
Hoobler, S. W. 711.
Hood, L. 198.
Hosoda, T. 234.
Hottle, G. A. 205.
Houck, J. C. 74, 579.
Houle, C. R. 126.
Howe, C. 420.
Hsiung, G.-D. 357.
Hsu, H. F. 467.
Hsu, S. Y. L. 467.
Huebner, R. J. 390.
Huggins, S. E. 231.
Hughes, F. W. 157.
Huls, H. N. 224.
Hundley, L. L. 372.
Hunt, V. R. 706.

Irvine, E. S. 629.
Israel, J. 479, 709.
Issekutz, B., Jr. 89.

Jacobson, L. O. 486.
Jankovic, B. D. 564.
Janoff, A. 354.
Jann, G. J. 671.
Jeffreys, C. L. 382.
Jernberg, N. A. 175.
Jirandoroski, J. 347.
Joachim, E. 665.
Johnson, B. C. 194.
Johnson, D. A. 375.
Johnson, E. J. 728.
Johnson, K. M. 56.
Johnson, M. K. 728.
Johnson, N. C. 518.

Kabakow, B. 569.
Kaelber, W. W. 375.
Kahn, R. H. 440.
Kaley, G. 354.
Kallmeyer, J. L. 198.
Kalmanson, G. M. 496.
Kampschmidt, R. F. 216.
Kaneko, T. 234.
Kaplan, D. 59.
Kare, M. R. 784.
Kartman, L. 700.
Katchalski, E. 661.
Kaufman, N. 22.
Ketler, A. 444.
Kind, L. S. 735.
King, J. S., Jr. 726.
Kingsbury, B. 99.
Kinney, T. D. 22.
Klavins, J. V. 22.
Klein, A. 82.
Knigge, K. M. 18.
Knight, V. 56.
Knudsen, P. J. 591.
Koenig, S. 755.
Koike, M. 315.
Korzenovsky, M. 637.
Koutras, G. A. 48.
Kramer, S. 535.
Kritchevsky, D. 254.
Krivoy, W. 134.
Kroeger, D. C. 134.
Krupp, M. A. 592.
Kuck, N. A. 34.
Kuhns, W. J. 63.
Kuron, G. W. 214.
Kuroyanagi, Y. 771.
Kuypers, H. G. J. M. 634.
Kwong, E. 468.

Lampen, J. O. 170.
Laneri, A. V. 111.
Lang, S. 789.
Langille, J. 417.
Larson, W. M., Jr. 140.
Law, L. W. 715.
Layton, L. L. 623.
Leak, D. 597.
Leathem, J. H. 113.
Lee, L. T. 420.
Lee, R. 321.
Lee, S. 623.
Leikhim, E. J. 454.
Leonard, S. L. 224.
Leveille, G. A. 313.
Levenson, S. 479, 709.
Levine, S. 109.
Lewis, A. M., Jr. 219.
Lewis, J. H. 382.
Liebelt, R. A. 377.
Lin, M. K. 27.
Lindstedt, S. 286.
Linkenheimer, W. H. 676.
Lochhead, A. C. 535.
Loebl, W. Y. 661.
Long, E. V. 689.
Loosli, J. K. 77, 468.
Lorenzen, I. 325.
Lowe, M. 549.
Ludwig, W. J. 703.
Lythgoe, J. P. 760.

Maca, R. 282.
MacCready, R. A. 805.
Machlin, L. J. 819.
Machlowitz, R. A. 508.
MacKenzie, M. 641.
Mackie, J. D. 649.
Malins, D. C. 126.
Maplesden, D. C. 77.
Marks, E. K. 486.
Marx, W. 674.
Masoro, E. J. 609.
Mattick, L. R. 468.
May, K. N. 185, 189.
Mayor, H. D. 103.
McCarthy, F. J. 723.
McCoy, T. A. 629.
McDonald, T. P. 428.
McElroy, W. T., Jr. 89.
McGarrahan, J. F. 117.
McKee, R. W. 140.
McKinney, G. R. 273.
Meeker, W. R., Jr. 774.
Meites, J. 440.
Meyer, H. M., Jr. 200.
Meyer, K. 59.
Midgley, A. R., Jr. 85.
Miller, C. P. 183.
Miller, F. 539.
Miller, K. D. 117.
Mirand, E. A. 360.
Mitchell, W. L. 16.
Moewus, L. 762.
Moloney, J. B. 715.
Moncloa, F. 336.
Montgomery, P. O'B. 372.
Moore, A. E. 409.
Moore, B. 53.
Morales, F. 522.
Morris, J. A. 56.
Moses, C. 559.
Mosley, J. W. 406, 524.
Moynihan, J. L. 254.
Murdaugh, H. V., Jr. 16.

Nabb, D. P. 512.
Nakagawa, Y. 401.
Nakamura, M. 315.
Nakatini, M. 315.
Nandi, S. 1.
Nath, M. C. 337.
Necheles, H. 771.
Nelson, D. L. 24.
Nichol, C. A. 96.
Nichols, G., Jr. 228.

Nicoll, C. S. 440.
Nimni, M. E. 38.
Nocenti, M. R. 546.
Nordsiek, F. W. 692.
Norton, H. W. 194.

Oates, J. A. 264.
O'Dell, B. L. 402, 512.
Odell, T. T., Jr. 428.
Okuda, K. 737, 816.
Oldstone, M. 709.
O'Malley, J. P. 200.
Opel, H. 291.
Orbison, J. L. 786.
Osterberg, K. A. 300.
Ott, W. H. 214.
Overholt, E. L. 732.
Overman, J. R. 219.

Pace, D. M. 282.
Palmer, J. G. 493.
Palmer, R. F. 296.
P'an, S. Y. 680.
Papaevangelou, G. J. 505.
Papermaster, B. W. 79.
Parker, R. B. 749.
Paterson, P. Y. 768.
Patt, H. M. 808.
Paulsson, J.-E. 385.
Payne, H. G. 273.
Pearson, B. 619.
Peck, W. A. 786.
Perreault, M. 479.
Perry, J. F., Jr. 522.
Perry, S. 615.
Phillips, P. H. 829.
Pierce, G. B., Jr. 85.
Pipes, G. W. 144.
Pogo, B. G. T. 409.
Popp, R. A. 561.
Porter, E. 609.
Prince, R. K. 574.
Powers, J. J. 185, 189.
Pratt, D. E. 189.
Prentice, T. C. 360.
Pressman, D. 347.
Pretell, A. 336.
Proctor, J. F. 77.

Rabinovici, N. 752.
Radford, H. M. 604.
Raines, M. 786.
Rambach, W. A. 793.
Ramey, E. R. 74.
Ramos, A. 129.
Rampone, A. J. 278.
Rapaport, S. I. 115.
Reardon, J. Z. 684.
Regan, W. O. 512.
Reichlin, S. 789.
Reynolds, G. 402.
Reynolds, S. R. M. 649.
Richmond, C. R. 797.
Ritts, R. E. 166.
Roantree, R. J. 654.
Root, H. D. 522.
Rose, H. M. 420.
Rose, J. C. 686.
Rose, K. D. 282.
Rosen, F. 96.
Rosen, L. 474.
Rosenkrantz, H. 417.
Rosenthal, F. 267.
Rosenthal, S. M. 501.
Rosevear, J. W. 276.
Rothenberg, S. P. 45.
Rowe, W. P. 390.
Rulon, O. 380.
Rutenburg, S. 261.
Ryan, R. J. 395.

Sachs, M. L. 254.
Salido-Rengell, F. 67.
Salvin, S. B. 498.
Samachson, J. 308.
Sams, W. M., Jr. 533.
Sauberlich, H. E. 313, 544.
Savage, J. E. 402.
Sawyer, C. H. 154.
Scala, A. R. 766.
Schartum, S. 228.
Schilling, R. F. 48.
Schneyer, C. A. 584.
Schneyer, L. H. 584.
Schrek, R. 328.
Schweinburg, F. B. 479.
Schwenk, E. 665.
Seabury, J. C. 16.
Sela, M. 661.
Sendroy, J., Jr. 641.
Sethi, P. 74.
Severson, A. R. 572.
Shafer, W. G. 694.
Shelokov, A. 823.
Sherins, R. 735.
Shetlar, M. R. 401.
Shirley, B. 629.
Shockley, J. W. 313, 544.
Shoemaker, W. C. 248.
Sieker, H. O. 16.
Sigel, M. M. 762.
Simmons, E. L. 486.
Simpson, W. F. 434.
Sirota, J. H. 592.
Sjoerdsma, A. 264.
Skillen, R. G. 121.
Slapikoff, S. 569.
Slater, C. R. 140.
Slater, J. V. 198.
Smadel, J. E. 200.
Smith, J. G., Jr. 533.
Smith, R. F. 498.
Sniffen, E. P. 464.
Snyder, M. L. 749.
Sokoloff, L. 792.
Solish, G. I. 645.
Spector, B. 146.
Spencer, H. 308.
Spicer, D. S. 508.
Spitzer, J. J. 89.
Sprunt, K. 755.
Steenbock, H. 105.
Steigleder, G. K. 59.
Steinetz, B. G. 113.
Steward, J. P. 654.
Strain, L. 121.
Straub, W. H. 686.
Straube, R. L. 808.
Strömblad, B. C. R. 345.
Stulberg, C. S. 434.
Su, S.-C. 457.
Sugiyama, H. 92.
Sullivan, J. M. 686.
Sulman, F. G. 342.
Suntzeff, V. 53.
Sussman, L. 569.
Sutton, D. D. 170.
Sweeney, E. E. 671.
Swell, L. 810.
Swenson, O. P. 24.
Swigart, R. H. 340.

Taquini, A. C., Jr. 711.
Tashian, R. E. 364.
Teller, W. M. 276.
Tennent, D. M. 214.
Thienes, C. H. 121.
Thompson, R. C. 296.
Tierney, J. 780.
Timiras, P. S. 267.
Tindall, J. P. 533.
Tobian, L. 615.
Tobias, G. J. 592.
Tocantins, L. M. 689.
Tolbert, O. 412.
Torii, S. 315.
Torop, H. A. 723.
Trager, W. 175.
Travis, R. H. 99.
Treadwell, C. R. 810.
Tregier, A. 152.
Tulis, J. J. 732.
Turner, C. W. 144, 212, 627.
Tytell, A. A. 508, 723.

Ullmann, T. D. 661.
Upton, A. C. 464.
Usdin, E. 457, 461.
Usdin, V. R. 457, 461.
Ushiyama, J. 520.
Uzman, L. L. 32.

van den Noort, S. 32.
Van Orden, F. 372.
Verder, E. 501.
Vick, J. A. 24.
Vogel, J. J. 829.
von Berswordt-Wallrabe, R. 144, 212.

Waddell, W. R. 760.
Wainfan, E. 674.
Waisman, H. A. 332.
Waldmann, T. A. 425.
Walford, R. L. 149.
Walker, R. I. 493.
Wang, H. L. 332.
Watson, D. W. 79.
Wattenberg, L. W. 300.
Weber, G. 631.
Weigle, W. O. 85.
Weinstein, L. 471.
Weiss, H. S. 242.
Weller, J. M. 209.
Wender, S. H. 401.
Wenk, E. J. 109.
Werner, S. C. 780.

Whitney, J. E. 12.
Whittle, W. 629.
Wiener, E. 752.
Wilpizeski, C. 319.
Wilson, J. C. 366.
Wittmers, L. E. 24.
Wiznitzer, R. G. T. 261.
Wolff, F. F. 464.
Wood, W. A. 162.
Woods, W. D. 786.
Worthen, D. M. 24.
Wright, G. G. 740.
Wright, L. D. 600.

Yajima, H. 559.
Yang, Y. T. 776.
Yaron, A. 661.
Yeager, V. L. 572.
Yesair, D. W. 119.
Youngner, J. S. 505.
Yull, A. B. 252.

Zanettti, M. E. 214.
Zweifach, B. W. 27.

SUBJECT INDEX

VOLUME 108

(*The numerals indicate the page*)

Accommodation, minimal gradient. 520.
Acid, acetic, acetate, ruminant. 194.
  amino-, 5-hydroxytryptophan, serotonin. 264.
    urinary, excretion, rat. 105.
  bile, see **Bile** acid.
  fatty, depletion, synthesis. 819.
    monoenoic, dogfish liver. 126.
    uptake by muscle after DNP. 89.
  folic, leukemia cells. 409.
  formiminoglutamic, excretion, vitamin B_{12} index. 703.
  mevalonic, not isovalthine precursor. 600.
    steroid-C^{14} fecal excretion. 555.
  mucopolysaccharides, gargoylism. 276.
  nucleic, metabolism, bone marrow. 366.
    precursors, inhibition, aminonucleoside nephrosis, rat. 706.
  polyglutamic, multichain, plasma expander. 661.
  ribonucleic, poliovirus, infectivity. 755.
    skin homografts. 549.
  sialic, epiphyseal plate, rabbit. 111.
    Escherichia coli. 34.
Adrenal cortex, response to stress, rats. 18.
    secretion, nephrectomy. 99
  glucocortoids, bone. 228.
  tissue, DOC hypertension. 615.
Aging effects, bone sodium, acute changes, serum sodium. 697.
    kidney. 808.
  liver hemodynamics. 752.
Alcohol, caffeine, rats. 157.
Aldehyde blocking, lathyrogenic agents. 572.
Algae, nystatin inhibition. 170.
Allergy, human, diagnosis, passive sensitization, monkey. 623.
Anaphylaxis, passive cutaneous, mouse, chicken. 129.
Antibiotic feeding, X-irradiation. 676.
  nystatin, yeast. 170.
  pretreatment, shock, rats. 27.
Antibody formation, actinophage injection, mice. 79.
  fragment III, complement. 454.
  hemagglutination-inhibition, adenovirus infections. 474.
  hepatitis, A-1 virus. 200.
Antigen elimination, Arthus phenomenon, guinea pig. 48.
  skin-testing, *C. neoformans.* 498.
Antigenicity, liver cell fractions. 234.
Antitoxin, human diphtheria, disappearance from skin. 63.
Aorta maturation, cholesterol synthesis. 257.
Arthus phenomenon, antigen elimination, guinea pig. 48.
Ascites, pertussis vaccine. 109.
Atherosclerosis, cholesterol-fed cockerels, estrogen. 786.

Bacterium, *Aerobacter aerogenes,* growth, cholesterol solubility. 674.
  Bacillus anthracis, lysozyme susceptibility. 740.
  enteric, mouse, peritoneal fluid. 654.
  Escherichia coli, sialic acid. 34.
  myco-, carbohydrate testing. 671.
  Salmonella infection, resistance. 512.
  Staphylococcus, endotoxin-like components. 166.
Bile acid binders, plasma cholesterol. 214.
    excretion, experimental hyperlipemia. 337.
    synthesis, mevalonic acid. 555.
    turnover, man. 286.
  intrinsic factor, vitamin B_{12} complex, intravenous injection. 737.
Blood, body weight, albino rat, antiproteolytic power. 658.
  cell, red, strangulation fluid. 252.
  coagulation, plasma thromboplastin antecedent. 115.
  erythrocytes stroma, eosinophil stimulation. 566.
    human, esterases. 364.
  erythropoiesis stimulation, pheochromocytoma. 425.
    tissue homogenates. 793.
  erythropoietin, unilateral renal ischemia. 546.
  gamma globulin, rabbit, guinea pig complement. 454.
  hemodynamics, liver age. 752.
  leukemia, Friend, murine hosts. 826.
      virus, chemotherapy. 360.
    Myleran, mouse thymus. 464.
    virus, congenital transmission, mouse. 715.
  leukocyte, Echo 9 virus. 742.
    interferon production. 799.
    labelling, inorganic P^{32}. 493.
  nucleotides, tolbutamide. 192.
  plasma, prothrombin activation, nonenzymic. 117.
  platelets, clotted thromboplastin. 382.
    in vivo transfer, thrombopoietic factor. 146.
    production, serum injection. 428.
  renal dynamics. 122.
  vessels, human, 24-dehydrocholesterol. 43.
    large human, thromboplastic, fibrinolytic activity. 369.
  volume parameters, poikilotherms. 231.
  withdrawal, hematocrit. 242.
Body metabolism, cat, hypothalamic lesions. 375.
  weight, blood, albino rat, antiproteolytic power. 658.
    gain, normal, goitrogenic diet. 692.
  adrenal effects. 228.
  marrow, nucleic acid metabolism. 366.
    transplantation, induced tolerance, mouse sex. 689.
Brain, 5-hydroxytryptamine ozone, rat. 121.
  irritative lesions, pituitary stimulation. 604.
  peptidases, metals. 32.
Burns, infection, mice. 501.

Calcium, intestinal absorption. 296.
Callus, fracture, hexosamine, hydroxyproline. 70.

Cancer, liver, phagocytosis. 216.
  resistance, mice. 140.
  serum enzyme alterations. 569.
Carbohydrate testing, mycobacteria. 671.
Cataract development, quercetin-fed rat. 401.
Cell culture, automatic: cytostat. 385.
    mouse, mouse hepatitis virus. 524.
    species antigens, immunofluorescence. 434.
    sterol synthesis. 282.
  immunologically active, ribonuclease. 564.
  living, nuclei, ultraviolet irradiation. 372.
  odontoblast, membrane potentials. 134.
  "sex," viral growth. 471.
Chlorpromazine metabolism, species dependence. 178.
Cholesterol, adrenal, methylandrostenediol. 113.
  aorta maturation, chicken. 257.
  biosynthesis, pregnant rat. 665.
  -fed cockerels, atherosclerosis, estrogen. 786.
  from bile acids. 286.
  level, rat tissue, lipid unsaturation. 637.
  metabolism, mevalonic acid. 555.
  serum, see **Serum** cholesterol.
  solubility, bacterial growth. 674.
  synthesis inhibitors. 214.
  thyroid effect, rat. 254.
  24-dehydro-, human blood vessels. 43.
  vitamin B_{12}. 119.
Cold acclimation, vitamin A. 609.
Contraceptions, progestins. 3.
Copper deficiency, chick, connective tissue. 402.
  metabolism, pregnancy. 518.
Cryptococcus neoformans, skin-testing antigen. 498.
Culture medium, automatic change. 175.
Cytopathic changes, non-transmissible, Sendai virus. 303.
Cytotoxicity, adrenal cortex hormones, lymphocytes. 328.

Dental caries assay. 829.
Diet, magnesium deficient, rat mitochondria. 315.
  plasma lipid relationships. 313.
  potassium, Cs^{137} excretion, rat. 797.
  protein synthesis, tryptophan deficiency. 38.
Diuresis, postprandial, seal. 16.
Drug, anti-estrus, adrenal subestrus. 514.
  LSD-25, food intake, rat. 319.
  salicylate, prophylactic counter-irritation. 432.
Dystrophy, muscular, rabbit. 77.

Electrolytes, sodium chloride, rats. 209.
Emetic sites, enterotoxin, monkey. 92.
Encephalomyelitis, allergic, cellular transfer, splenectomized albino rat. 768.
Enzyme, cholinesterases, psychotropic compounds. 457.
    serum. 71.
  co-, Q, proliferative lesions, liver. 300.
  esterases, multiple forms, human erythrocytes. 364.
  glucosidase, mammalian, histochemical demonstration. 619.
  hyaluronidase, trypsin inhibitors, urine. 591.
  kidney, liver, glucose-6-phosphatase, 6-phosphogluconate dehydrogenase, lactic dehydrogenase, phosphohexoseisomerase. 631.
  lysozyme, *Bacillus anthracis* susceptibility. 740.
  mono-amine oxidase inhibition, hypoglycemia, angina pectoris. 597.
    inhibitor. 680.
    psychotropic compounds. 461.
  neuraminidase, hemagglutinin, influenza viruses. 420.
  peptidase, brain, metals. 32.
  phosphatase, prostatic acid, canine. 417.
    human serum. 53.
  phosphorylase, denervated muscle. 224.
  pitocinase, sheep gravid uterus. 649.
  ribonuclease, immunologically active cells. 564.
  serum, alterations, cancer. 569.
  transaminase, alanine, DOC effect. 96.
  trypsin, inflammation. 579.
   inhibitor, Shwartzman phenomenon. 587.
   measles virus. 508.
  xanthine oxidase, liver. 22.
Eosinophil-stimulating substance, erythrocyte stroma. 566.

Fat absorption, lymphatics. 278.
  isomerized, serum cholesterol. 468.
Fertility, increased, vitamin B_{12}. 380.
  semen, frozen rabbit sperm. 663.
Fibers, cortico-cuneate. 634.
Flea-bite, skin reactivity, guinea pig. 700.
Fluoride metabolism, pregnant rat. 342.

Gargoylism, mucopolysaccharide excretion. 276, 529.
Gland, salivary, iodine-induced sialadenitis. 136.
Glucose metabolism, alternate pathways. 728.
  release, hepatic potassium. 248.
  uptake stimulation, tumor extracts. 12.
Goiter, diet, normal body weight gain. 692.
  production, Tapazole, thyroxin secretion. 627.
Growth, hair, rabbit, mucopolysaccharides. 59.

Heart, left ventricle, infarct, teflon prothesis. 82.
Hemagglutination inhibition, encephalomyocarditis virus. 823.
Hematocrit, maximal, sex differences. 340.
  sampling. 242.
Hexosamine, hydroxyproline alterations, sun-damaged skin. 533.
   fracture callus. 70.
Homograft, skin, rat, ribonucleic acid extracts. 549.
Hormone, adrenal cortex, lymphocytes. 328.
   dermal response. 74.
  ACTH release, hypothalamic lesion. 377.
   synthetic tricosapeptide. 559.
  androgen, anabolic-androgenic activities. 273.
  cortisone, 9α-fluorohydro-, 2-methyl-9α fluorohydro-, renal clearance. 592.
  deoxycorticosterone, inhibition ACTH release. 96.
  estrogen, atherosclerosis, cholesterol-fed cockerels. 786.
   -treated male rat. 395.
  gastrin, depletion, reserpine, canine antral mucosa. 760.
  gonadotropin, chorionic, human, immunobiological identification. 85.
   pituitary, brain stimulation. 604.

  growth, avian, rat. 10.
  hydrocortisone, vaccinia virus. 67.
    venom, dog. 522.
  insulin effects, tissue catecholamines. 345.
  lactogenic, placentoma. 212.
    P^{32} uptake, pigeon. 144.
  norepinephrine metabolism, inhibition. 482.
  progestin enol ethers. 3.
    ovarian blood, rabbit. 154.
  prolactin production, rat pituitary, *in vitro*. 440.
  response, mammary tissues. 1.
  serotonin, 5-hydroxytryptophan. 264.
    lung, increased, mice, antiserum to Ehrlich's ascites tumor. 735.
    release, bacterial endotoxin. 774.
  therapy, pancreas, hypophysectomized rat. 238.
  thyroxin concentration, pituitary-thyroid regulation. 789.
    D-, vascular connective tissue. 325.
    secretion, fowl, goitrogens. 627.
Hyperlipemia, experimental, bile acid excretion. 337.
Hypertension, DOC, adrenal tissue. 615.
  pyelonephritis. 496.
Hyperthermia, hypothermia, blood volume, alligators. 231.
Hypophysis, see **Pituitary.**
Hypothalamus, lesion, ACTH release. 377.
  septal & anterior lesions. 375.
  stimulation, ovulation. 291.
Hypothermia, mouse, influenza virus infection. 776.

Immune response, actinophage, mouse. 79.
    Peyer's patch protection. 487.
  serum, hemorrhagic shock. 479.
Immunofluorescence, mammalian cells. 434.
Immunohistochemical identification, tissue culture cells. 347.
Infection, mice, burns. 501.
  red blood cell. 252.
Inflammation, chemistry. 579.
Interferon production, leukocytes. 799.
    non-transmissible cytopathic changes. 303.
Iodine-induced sialadenitis. 136.
Iron metabolism, liver. 22.
Irradiation, Peyer's patch protection. 487.
  ultraviolet, living cell nuclei. 372.
Isotope, C^{14}-phytosterols, metabolism. 810.
  Cs^{137} excretion, rat, dietary potassium. 797.
  P^{32}, inorganic, leukocyte labelling. 493.
    uptake, pigeon, lactogenic hormone. 144.
  Yttrium^{91}, localization, *Perognathus penicillatus*. 198.

Kidney, carbohydrate metabolism. 631.
  cell cultures, monkey, mycoplasma (PPLO), hemadsorption. 616.
    infectious hepatitis. 813.
  dynamics, blood. 122.
  M-protein localization 539.
  nephritis, pyelo-, hypertension. 496.
  renin, angiotensinase content, normal, renal hypertensive rats. 711.
  17 OHCS secretion, ACTH injection. 99.
  steroid effects. 592.
  unilateral ischemia, erythropoietin. 546.
  X-irradiation effects. 808.

Lipid in lipoproteins. 544.
  metabolism, tissue culture. 6.
  unsaturation, tissue cholesterol levels. 637.
Lipids, plasma, see **Plasma** lipids.
Liver, carcinogenesis, reticuloendothelial system. 216.
  cell fractions, antigenic properties. 234.
  dogfish, monoenoic fatty acids. 126.
  endotoxin clearance. 261.
  function, rat, radioiodinated antipyrine measurement. 686.
  hemodynamics, age. 752.
  hepatitis, infectious, kidney cells. 813.
  mitochondria, heart mitochondia, swelling, magnesium deficient rat. 315.
  potassium, glucose release. 248.
  proliferative lesions, coenzyme Q. 300.
Lupus erythematosus, serum reaction, DND. 149.

Magnesium deficiency, rat, mitochondria. 315.
  metabolism, digitoxin. 684.
Mammary tissue response, somatotropin-containing hormone. 1.
Microorganisms, interaction, measurement. 749.
Muscle, accommdation, minimal gradient. 520.
  denervated, phosphorylase. 224.
  skeletal, free fatty acid uptake, DNP. 89.

Nephrosis, rat, aminonucleoside. 706.
    -8-C^{14} metabolism. 611.
Nicotine oxidation, *Arthrobacter*. 162.

Ovariectomy, RES response. 354.
Ovary, rabbit, progestin release. 154.
Ovulation, hypothalamic stimulation. 291.

Pancreas, hypophysectomy, rat. 238.
Peptide, synthetic, ACTH activity. 559.
Peritoneal fluid, mouse, enteric bacilli. 654.
Phenylketonuria, experimental, rats. 332.
Pituitary, ACTH release, DOC inhibition. 96.
  extracts, tibial growth, blood. 10.
  gonadotropin release, brain stimulation. 604.
  pancreas. 238.
  prolactin *in vitro*. 440.
  -thyroid regulation, thyroxine concentration. 789.
Plaque assay technique, bottle cultures. 406.
  production, polivirus, temperature, L-cystine. 412.
Plasma, erythrocyte cholinesterase inhibition. 457.
  expander, multichain polyglutamic acid. 661.
  gamma globulins, specificity, rheumatoid factors. 744.
  human, heme synthesis. 535.
  lipids, relationships, dietary factors. 313.
  myco-, (PPLO), hemadsorption, MKC cultures, rats. 616.
  thromboplastin antecedent levels, coumarin anticoagulants, Laennec's cirrhosis. 115.
Polysaccharide, muco-, hair growth, rabbit. 59.
Potassium deficiency, rat salivary gland. 584.
  dietary, rat, Cs^{137} excretion. 797.
  fetal. 803.

  hepatic output, glucose release. 248.
Pregnancy, rat, fluoride metabolism. 342.
Prostate, canine, acid phosphatase. 417.
Protein, desoxyribonucleo-, fibrous, non-fibrous. 149.
  lipo-, lipid distribution. 544 .
  M-, renal localization. 539.
  serum, see **Serum** protein.
  synthesis, tryptophan deficiency. 38.
Psychotropic compounds, enzyme systems. 457, 461.
Pulmonary edema. 735.

Quercetin, rat, cataract development. 401.

Radiation, tissue serotonin. 160.
Rat, Mann-Williamson. 771.
Reserpine, gastrin depletion, canine antral mucosa. 760.
Reticuloendothelial system, endotoxemia, immune serum. 479.
    hepatocarcinogenesis. 216.
    ovariectomy. 354.
Rheumatoid factors, specificity, gamma globulins. 744.

Salicylates, counter-irritation. 432.
Salivary gland, potassium-deficient rat. 584.
Schistosoma japonicum, circumoval test. 467.
Seal, volume receptors. 16.
Semen, fertility, frozen rabbit sperm. 663.
Serum, anti-, human chorionic gonadotropin. 85.
    -thyrotropin, hyperthyroidism, "abnormal activator". 780.
  cholesterol, isomerized fat. 468.
  cholinesterases. 71.
  human, prostatic phosphatase. 53.
  protein, calcium, reproductive cycle, pigeon. 641.
Sex, bone marrow transplantation, induced tolerance, mouse. 689.
  cell, viral growth. 471.
  differences, maximal hematocrit. 340.
  organs, male rat, estrogen-treated. 395.
Shock, bacterial factor. 27.
  hemorrhagic, immune serum. 479.
  vascular resistance. 24.
Shwartzman phenomenon, trypsin inhibitors. 587.
Skin reactivity, guinea pig, flea-bite. 700.
  site, transfer technic, antitoxin. 63.
  sun damage, hexosamine, hydroxyproline. 533.
Sodium, bone, changed serum sodium, effect of age. 697.
  dilantin, tissue culture. 694.
Spermatozoa, zinc. 321.
Steroids, urinary, man, high altitudes. 336.
Sterol synthesis, stress. 282.
  see also **Cholesterol.**
Strangulation fluid, red blood cell. 252.
Stress response, adrenal cortex, rat. 18.
Strontium, intestinal absorption. 296.
  radioactive (Sr^{85}), removal, man. 308.

Taste, vitamin A deficiency. 784.
Testis, orchiectomy, osteoarthritis, STR/IN mouse. 792.
Thymus, marrow cells. 561.
  mouse, lymphomas, Myleran. 464.
Thyroid, "abnormal activator," anti-thyrotropin sera. 780.
  cholesterol metabolism, rat. 254.
  estrous cycle. 484.
Tissue catecholamines, insulin effects. 345.
  connective, adrenal hormones, age. 74.
    cell transformation. 574.
    chick, copper deficiency. 402.
    thrysin, inflammation. 579.
    vascular, d-thyroxin. 325.
  culture cells, immunohistochemical identification. 347
    dental pulp. 134.
    dilantin sodium. 694.
    marine fish. 762.
  homogenates, erythropoiesis. 793.
  injury, ascorbate. 189.
    biochemical, physical changes. 185.
  serotonin, radiation. 160.
Tolbutamide, blood nucleotides. 192.
Toxin, endo-, bacterial, serotonin release. 774.
    hepatic clearance. 261.
    shock, vascular resistance changes. 24.
  entero-, staphylococcal, sites of action. 92.
  staphylococcal, rabbit, mechanism. 709.
Tumor, adrenal, anti-estrus drugs. 514.
  cells, sarcoma 180 ascites, Columbia SK virus. 244.
  development, transplanted nodules. 289.
  Ehrlich's ascites, antiserum injection, lung serotonin, pulmonary edema. 735.
  extracellular material. 629.
  extracts, glucose uptake stimulation. 12.
  lymphoma, mouse thymus, Myleran. 464.
  pheochromocytoma, erythropoiesis stimulation. 425.
  placentoma, lactogenic hormone. 212.

Urine, mucopolysaccharides, Hurler's syndrome. 529.
  normal, albumin, globulins. 726.
  preparations, hyaluronidase, trypsin. 591.
  streoids, man, high altitudes. 336.
Uterine cervix, secretions, isohemagglutinins. 645.
Uterus, gravid sheep, pitocin, pitocinase. 649.
  mouse, bacterial flora. 152.

Vaccine, live tularemia, aerogenic immunization. 732.
  pertussis, ascites production. 109.
Venom protection, hydrocortisone, dog. 522.
Virus, adeno-, HI antibody responses. 474.
  A-1, hepatitis. 200.
  bottle culture, plaque assay technique. 406.
  Columbia SK, S180 cells. 244.
  coryza-, common cold. 444.
  Echo 9, multiplication, human leucocytes. 742.
  encephalitis, western, Massachusetts. 805.
  encephalomyocarditis, cell "sex". 471.
    HI test. 823.
  hepatitis, mouse, cytopathogenicity, mouse cell cultures. 524.
  influenza, ammonium ion. 766.
    infection, hypothermia, mouse. 776.
  leukemia, congenital transmission, mouse. 715.
    Friend, chemotherapy. 360.
      labeled cells, folic acid. 409.

        murine hosts. 826.
    measles, trypsin. 508.
    myxo-, auto-inhibition, plaque formation. 357.
    neuraminidase. 420.
        parainfluenza type 2, plaques, monkey kidney cells. 723.
    polio, heat-resistance. 505.
        plaques, temperature, L-cystine. 412.
        ribonucleic acid, host cell. 755.
    polyoma, DNA. 103.
    pneumonia, mouse. 651.
    reo-, recovery, mouse. 390.
    Sendai, non-transmissible cytopathic changes. 303.
    SV_{40}, human volunteers. 56.
    tumor development. 289.
    vaccinia, hydrocortisone, topical application. 67.
        non-infective, eggs. 219.
    vacuolating, formaldehyde. 205.

Vitamin A, cold acclimation. 609.
        deficiency, taste. 784.
    B_{12}, assay. 45.
        cholesterol. 119.
        complex, bile intrinsic factor, intravenous injection. 737.
        deficiency, excretion, formiminoglutamic acid. 703.
        fertilizability, sand dollar eggs. 380.
        intestinal absorption, D-sorbitol. 816.
    D, urinary amino acid excretion, rat. 105.
    E, selenium, muscular dystrophy, rabbit. 77.

X-Radiation, antibiotic feeding. 676.
    electroshock convulsions. 267.
    experimental infection, mice. 183.
    kidney growth. 808.

Zinc metabolism, pregnancy. 518.
    rat fertility. 321.

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The Wm. S. Merrell Co.
Miles Laboratories, Inc.
Ortho Pharmaceutical Corporation
Parke Davis and Co.
Chas. Pfizer and Co., Inc.
Pitman Moore Co.
Riker Laboratories, Inc.
The Rogoff Foundation
Damon Runyon Memorial Fund
Sandoz Pharmaceuticals
Schering Corporation
G. D. Searle and Co.
Sharp and Dohme, Division of Merck & Co., Inc.
Smith Kline and French Laboratories
Smith, Miller & Patch, Inc.
Squibb Institute for Medical Research
Sterling-Winthrop Research Institute
G. N. Stewart Memorial Fund
U.S. Vitamin Corporation
The Upjohn Co.
Wallace Laboratories, Division of Carter Products, Inc.
Warner-Chilcott Laboratories
The Wilson Laboratories
Wyeth Laboratories
Anonymous